Clinical pharmacology of tuberculosis drugs and tuberculosis control in developing world

Vu Dinh Hoa

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The Involvement of Private Pharmacy and the Individualization of Treatment using Dried Blood Spot

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Chapter 1

General Introduction and Objective of the Thesis

Tuberculosis (TB) is a communicable disease caused by Mycobacterum tuberculosis, which occurs worldwide with a high burden in the developing countries. Successful treatment has been reported for decades and cumulative knowledge about this disease is available nowadays. Still, even in the early 21st century TB remains a high-burden infectious disease with high morbidity and mortality. One-third of the world population is infected with Mycobacterium tuberculosis in its dormant state. About 9 million of those infected develop the disease, and this number increases annually (1). This brings up questions as to whether our current strategy to control this disease is the best one. It is not easy to find answers but two subjects need to be addressed in the fight against TB. First, TB is easily transmitted in the community, which challenges our effort to prevent its spreading. TB is often detected late, resulting in late confirmation of the right diagnosis. This increases the risk of infecting many other individuals in the community (2). Failure to recognize the infection in time results in late initiation of treatment or no treatment at all. Second, TB treatment faces many difficulties, the most worrisome of which are multi-drug resistance/extensively drug resistance (MDR/XDR) TB and the combination of TB-HIV. Drug-drug interactions and high risk of toxicity resulting in non-adherence complete the challenge of treatment optimization.

TUBERCULOSIS CONTROL IN PRIVATE PHARMACIES

In the developing world, TB control is challenged by many problems. The lack of health care facilities and human resources prevent the National Tuberculosis Program (NTP) from establishing a comprehensive system for this purpose. Vietnam is one of the countries to have successfully established a TB control system with a high political commitment that covers the whole country, including remote areas (3). Vietnam achieved the target of the World Health Organization for TB diagnosis and successful treatment for about 10 consecutive years, yet the notification rate did not decrease as desired (3-5). The potential reason is that substantial proportions of patients were diagnosed late or were not detected at all. This resulted in the transmittance of *M. tuberculosis* to other people even before diagnosis. TB patients in Vietnam, like most low- and middle-income countries, were treated not only in the public sector as NTP but also in the private sector. Previous data suggests that private pharmacies are most likely the source causing the delay in diagnosis and the ensuing treatment (4,5). No clear explanation has been found as to why this factor is the main cause of diagnostic delay to date. Pharmacists who are in charge of private pharmacies may provide the answer for this question. So far, the private sector is still out of NTP's understanding and efforts have been made to establish collaboration between the two sectors in the form of a public-private mix (6). Incorporating private pharmacies into this program proved to be a successful approach to increase case detection (7). Therefore, the aim of this thesis was to explore the role of private pharmacies in tuberculosis control in a complex setting (Hanoi) of a developing country (Vietnam).

TUBERCULOSIS TREATMENT OPTIMIZATION

To date, TB treatment follows standardized dosing. The main idea behind this is to reach efficacy in a majority proportion of patients with a minimum of toxicity. This approach is an economic and easy way to achieve successful treatment outcome in the general patient population (1,8). However, in daily practice physicians and clinical pharmacists handle individual patients and not all of them fit this approach. For those patients, individualized treatment by means of therapeutic drug monitoring (TDM) is highly recommended to ensure enough exposure for adequate efficacy and to avoid toxicity (9-16). Studies based on in vitro models and clinical data on anti-TB drugs suggest that inter-patient

variability plays an important role in the emergence of resistance and failure (17-19). Assessing drug concentration by means of TDM could help assure adequate drug exposure while reducing the risk of toxicity. TDM may have the additional benefit of helping physicians cope with disease-drug interaction, drug-drug interaction and non-adherence. Implementing traditional plasma sampling for TDM faces many hurdles, like lack of sample stability and the need for venipuncture to collect the sample. This may be one reason why TDM has not yet been implemented in NTP. A new applied procedure in the field of pharmacokinetics is dried blood spot (DBS) sampling, which has been used to screen metabolic diseases among infants for decades (20). A dried blood spot (DBS) is obtained from blood by a finger prick, then put on an absorbing paper. With the advantage of high stability, lower blood volume, easy sampling and lower biohazard risk, DBS is an attractive alternative specimen that can make TDM possible, even in a resource-limited area (21). DBS has already been applied in the sampling procedure for many drugs such as immunosuppressants, antiretroviral agents and antimalarial drugs. Peculiarly, available data on DBS for anti-TB drugs are scarce (22). Therefore, the aim of this thesis was to develop bioanalytical methods that are useful for TDM of anti-TB drugs using DBS sampling. In addition, the requirements, obstacles and solutions for developing and applying dried blood spot method for TB drugs were assessed.

OBJECTIVE OF THE THESIS

To summarize, the objectives of this thesis were to explore the role of private pharmacies and to develop and evaluate DBS sampling for anti-TB drugs both as potential new additives for improving TB control and treatment outcome.

REFERENCES

(1) World Health Organization. Global tuberculosis report 2012. Genever, Switzeland: WHO: World Health Organization; 2012.

(2) Dye C, Williams BG. The population dynamics and control of tuberculosis. Science 2010 May 14;328(5980):856-861.

(3) Huong NT, Duong BD, Co NV, Quy HT, Tung LB, Bosman M, et al. Establishment and development of the National Tuberculosis Control Programme in Vietnam. Int J Tuberc Lung Dis 2005 Feb;9(2):151-156.

(4) Huong NT, Vree M, Duong BD, Khanh VT, Loan VT, Co NV, et al. Delays in the diagnosis and treatment of tuberculosis patients in Vietnam: a cross-sectional study. BMC Public Health 2007;7(1471-2458):110.

(5) Lonnroth K, Thuong LM, Linh PD, Diwan VK. Delay and discontinuity-a survey of TB patients' search of a diagnosis in a diversified health care system. Int J Tuberc Lung Dis 1999 Nov;3(11):992-1000.

(6) Lonnroth K, Karlsson M, Lan NT, Buu TN, Dieu TT. Referring TB suspects from private pharmacies to the National Tuberculosis Programme: experiences from two districts in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 2003 Dec;7(12):1147-1153.

(7) Quy HT, Lan NT, Lonnroth K, Buu TN, Dieu TT, Hai LT. Public-private mix for improved TB control in Ho Chi Minh City, Vietnam: an assessment of its impact on case detection. Int J Tuberc Lung Dis 2003 May;7(5):464-471.

(8) WHO global Tuberculsois.Stop TB Dept. Treatment of tuberculosis guidelines [WHO/HTM/TB/2009.420]. : World Health Organization; 2010.

(9) Magis-Escurra C, van den Boogaard J, Ijdema D, Boeree M, Aarnoutse R. Therapeutic drug monitoring in the treatment of tuberculosis patients. Pulm Pharmacol Ther 2012 Feb;25(1):83-86.

(10) Babalik A, Babalik A, Mannix S, Francis D, Menzies D. Therapeutic drug monitoring in the treatment of active tuberculosis. Can Respir J 2011 Jul-Aug;18(4):225-229.
(11) Alffenaar JW, Kosterink JG, van Altena R, van der Werf TS, Uges DR, Proost JH. Limited sampling strategies for therapeutic drug monitoring of linezolid in patients with multidrug-resistant tuberculosis. Ther Drug Monit 2010 Feb;32(1):97-101.
(12) Heysell SK, Moore JL, Keller SJ, Houpt ER. Therapeutic Drug Monitoring for Slow Response to Tuberculosis Treatment in a State Control Program, Virginia, USA. Emerging Infectious Diseases 2010 OCT;16(10):1546-1553.
(13) Holland DP, Hamilton CD, Weintrob AC, Engemann JJ, Fortenberry ER, Peloquin CA, et al. Therapeutic Drug Monitoring of Antimycobacterial Drugs in Patients with Both Tuberculosis and Advanced Human Immunodeficiency Virus Infection. Pharmacotherapy 2009 MAY;29(5):503-510.
(14) Unsalan S, Sancar M, Bekce B, Clark PM, Karagoz T, Izzettin FV, et al. Therapeutic monitoring of isoniazid, pyrazinamide and rifampicin in tuberculosis patients using LC. Chromatographia 2005 JUN;61(11-12):595-598.
(15) Li J, Burzynski JN, Lee YA, Berg D, Driver CR, Ridzon R, et al. Use of therapeutic drug monitoring for multidrug-resistant tuberculosis patients. Chest 2004 12;126(0012-3692; 0012-3692; 6):1770-1776.
(16) Yew WW. Therapeutic drug monitoring in antituberculosis chemotherapy: clinical perspectives. Clin Chim Acta 2001 11;313(0009-8981; 1-2):31-36.
(17) Gumbo T. New susceptibility breakpoints for first-line antituberculosis drugs based on antimicrobial pharmacokinetic/pharmacodynamic science and population pharmacokinetic variability. Antimicrob Agents Chemother 2010 Apr;54(4):1484-1491.
(18) Pasipanodya JG, Srivastava S, Gumbo T. Meta-analysis of clinical studies supports the pharmacokinetic variability hypothesis for acquired drug resistance and failure of antituberculosis therapy. Clin Infect Dis 2012 Jul;55(2):169-177.
(19) Srivastava S, Pasipanodya JG, Meek C, Leff R, Gumbo T. Multidrug-resistant tuberculosis not due to noncompliance but to between-patient pharmacokinetic variability. J Infect Dis 2011 Dec 15;204(12):1951-1959.
(20) Guthrie R, Susi A. A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants. Pediatrics 1963;32(3):338-&.
(21) Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther Drug Monit 2009 06;31(3):327-336.
(22) Allanson AL, Cotton MM, Tettey JN, Boyter AC. Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: a potential method for therapeutic drug monitoring. J Pharm Biomed Anal 2007 08/15;44(0731-7085; 4):963-969.

Part I.

Tuberculosis control in private pharmacies in Vietnam

Chapter

2

Suspected Tuberculosis Case Detection and Referral in Private Pharmacies in Vietnam

Int J Tuberc Lung Dis. 2012 Dec; 16(12):1625–1629

Suspected tuberculosis case detection and referral in private pharmacies in Vietnam

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ABSTRACT

Settings: **Private Pharmacies in Hanoi, Vietnam.**

Objectives: **To explore the response of healthcare providers (HCPs) in private pharmacies to suspected tuberculosis patients.**

Methods: **A simulated patient method combined with an interview in 128 randomly selected private pharmacies and 10 private pharmacies nearby the tuberculosis hospitals.**

Results: **Fifty nine (46%) and 70 (55%) of HCPs referred the suspected tuberculosis to general healthcare in the simulated patient method and interview, respectively. Only 11 (9%) referred the simulated tuberculosis patient to a tuberculosis care facility. Fifty-two (42%) of the HCPs identified tuberculosis from a fictitious case described on paper; 34 (27%) were aware of the free treatment in the National Tuberculosis Program (NTP). Awareness of the free treatment in the NTP predicted a higher rate of direct referral to tuberculosis facilities (OR=5.80, 95%CI=1.88-19.62) and better ability to detect tuberculosis from a paper fictitious case (OR=5.14, 95%CI=2.36-11.73). Pharmacies certified as Good Pharmacy Practice (GPP) were less likely to refer simulated patients to tuberculosis facilities than non-GPP pharmacies (OR=0.10, 95%CI=≤0.01-0.79).**

Conclusions: **Nearly half of HCPs in private pharmacies do not refer suspected tuberculosis patients, possibly contributing to the delay in diagnosis and treatment. Awareness of the NTP's free treatment predicted better performance of HCPs.**

INTRODUCTION

Tuberculosis (TB) is a communicable disease occurring worldwide, 55% of global cases are notified in Asia. Vietnam is a high burden country, with an incidence of 150 000 TB patients per year (1). To reduce the burden of TB and associated morbidity and mortality, the World Health Organization (WHO) recommends the DOTS stategy . This strategy, emphasising case detection and standardised treatment both free of charge, was successfully implemented in Vietnam by the National TB Control Programme (NTP) (2,3). Hanoi is the second biggest city in Viet Nam, with population of approximately 3 million and an estimated TB prevalence of 146 per 100 000 population (3,4). TB cases are diagnosed using sputum smear examination and/or chest X-ray and, in some cases, sputum culture. Most facilities designated for TB diagnosis were established inside the NTP system; this includes the central TB hospital (the National Hospital for Lung Diseases) provincial TB hospitals/units and district TB units (3,4).

Private pharmacies constitute an important part of the private healthcare sector in many low- and middle-income countries (5,6). It is well known that private pharmacies also play a substantial role in diagnosis and treatment of TB (7-11). However, the link between private pharmacies and the public sector in TB control is still underdeveloped (3,12-14). Several studies have showned that in Vietnam private pharmacies were the most important cause of delays in diagnosing TBand, initiating TB treatment (15,16). Proposed causes are prescriptions by private pharmacies for suspected TB patients instead of referrals to designated health facilities for diagnosis. (9,11). However, it is not clear whether private pharmacies are unable to identify TB suspects or unwilling to refer them. Furthermore, factors that affect the performance of health care providers (HCPs) in private pharmacies are unknown. To explore the referral behaviour of HCPs towards TB suspects, we performed a study using a simulated patient method and interview in private pharmacies in Hanoi.

POPULATION AND METHODS

Setting and sample

A list of all 1308 private pharmacies registered in nine urban districts of Hanoi was provided by the respective District Health Bureaux. We aimed to achieve a 95% confi dence interval (95%CI) of ±0.08 for an estimated proportion of 0.5; the required sample size was therefore 135. A pilot survey of 20 pharmacies from this list revealed that half of the pharmacies could not be found at the address provided, and that of those found 70% (7/10) agreed to participate. To correct for these expected losses, 380 pharmacies were randomly selected from the list. The in-depth interview in the pilot study suggested that pharmacies located near the two TB hospitals in Hanoi (the National Hospital for Lung Diseases and Hanoi Tuberculosis and Lung Disease Hospital) may have different TB control activities. An additional sample including all pharmacies within 500 m of the two TB hospitals was therefore selected as a subgroup.

By 2010, private pharmacies in Hanoi were being upgraded and given Good Pharmacy Practice (GPP) certification due to changes in policy of the Ministry of Health. The GPP certificate was granted according to standardized criteria related to regulations, infrastructure, personnel and education; however, none of these focused on TB control. As the upgrading was still in progress at the time of this survey, both GPP and non-GPP pharmacies existed All HCPs from the selected pharmacies were asked to take part in a study on 'respiratory diseases'. They were informed that the study consisted of two parts that would be audio recorded: a visit by a simulated patient and an interview. Signed informed consent was

obtained for all participating HCPs. The study was approved by the Ethical Review Board of the Hanoi University of Pharmacy.

Design

The pharmacies first received a visit from a simulated patient, followed by an interview 1 week to 2 months later. The simulated patient method and interview were conducted by 10 trained research assistants (4 men and 6 women, age 23–26 years), who were students or lecturers from the Hanoi University of Pharmacy. The research assistant group was trained in 3 days to improve their knowledge of TB and interviewing skills, and participated in a role-playing workshop on the simulated patient method. For each pharmacy, the simulated patient and the interviewer were different to avoid possible bias during the case detection phase of the interview section. As pharmacies could have more than one HCP, the two research assistants ensured that the same HCP participated in both parts of the study.

The simulated patient method:

The simulated patient claimed to be suffering from cough and fever for 4 weeks. No improvement had occurred after two 10-day courses of antibiotics (amoxicillin followed by spiramycin). Furthermore, the simulated patient had been in contact with a TB patient and suggested that he/she might have contracted TB. Anti-tuberculosis drugs were requested. If additional questions were asked, the symptoms of mild fever, productive cough and no clear improvement with cough suppressants were mentioned. The 'patient' wrote down the decisions of the HCP and the drugs dispensed immediately after leaving the pharmacy.

The interview:

A structured questionnaire was developed using the adjustments from the pilot study. The interview consisted of general questions about the HCP and the characteristics of the pharmacy, followed by questions about a fictitious case on paper and knowledge about the NTP.

Fictitious case on paper:

A 50-year old man had been suffering from cough and fever for 4 weeks, for which he had taken a 10-day course of oral spiramycin with no improvement He visits your pharmacy and asks for your advice.

Q1: What would you do for this case?

If the HCP wanted to know more about the case, he/ she was informed that the patient suffered from mild fever, productive cough and had experienced no clear improvement using cough suppressants.

Q2: What disease/diseases do you think this patient is suffering from?

Knowledge about the National TB Control Programme

Q3: Are you aware of the NTP?

Q4: What are the costs of anti-tuberculosis drugs provided by the NTP?

The responses of the HCPs were noted immediately. The study design (simulated patient method followed by interview) is briefly presented in the Figure

Data collection, revision and analysis
Data collection forms were cross-reviewed by another research assistant with the help of the audiotapes, which were also used to ensure that for each pharmacy only one HCP participated in the study. All data collection forms were double-checked by a researcher and translated into English. A Microsoft Access 2007 (Microsoft Corp, Seattle, WA, USA) database was made, after which data analysis was performed in *R* 2.10.1 (GNU Project, Vienna, Austria, www.r- project.org).

To explore determinants for referral and the ability to identify TB cases by the HCPs, Firth's penalized logistic regression was performed using a stepwise approach (17). Categorical variables used in this analysis were transformed to dichotomous variables. The backward stepwise approach was performed as follows: the determination of potential covariates using univariate analysis with a cut-off *P* value of 0.2, building multivariate models consisting of potential covariates, and finally, the removal in succession of variables with no significant ($P > 0.05$) or confounding effect (changes in odds ratios [ORs] of the remaining variables of <15%).

RESULTS

Of the list of 1308 pharmacies, 380 were randomly selected; 206 were found (54%), of which 129 (63%) agreed to participate in the study. One record was lost due to technical problems. Data from 128 pharmacies were thus analysed as the main group. Among 20 pharmacies located close to the TB hospitals, three were already selected for the main group and seven refused to participate, leaving a subgroup of 10 (50% of 20) pharmacies. A total of 138 pharmacies were included in the study.

GPP certification was obtained by 30 (23%) pharmacies in the main group. Most participating HCPs were assistant pharmacists who had received 2 years' training (67%), 17% were drug sellers who had received 6 months' training and 9% were pharmacists who had completed a 5-year pharmacy programme at the university level. Medians (interquartile ranges) of the HCPs' age and working experience in the pharmacies were respectively 26 (23–37) and 4 (2–10) years. A similar pattern was observed in the subgroup (Table 1).

When confronted by the simulated patient, 68 (53%) HCPs decided to dispense drugs, none of which were anti-tuberculosis drugs; 59 (46%) HCPs referred the patient for diagnosis: of these, 48 (38%) referred the patient to general health care and 11 (9%) to a designated TB facility. Responses to the fictitious case on paper by 58 (45%) HCPs were to sell drugs and by 70 (55%) HCPs to refer to health care services.. Drugs suggested by the HCPs for the fictitious patient on paper did not include anti-tuberculosis agents. The diseases most frequently mentioned were pneumonia (45%), TB (40%) and bronchitis (39%). HCPs mentioned TB as a possible disease and were considered to be able to identify TB from the fictitious case on paper. One hundred and three (80%) respondents said that they were aware of the NTP; of these, 34 (27%) knew that anti-tuberculosis treatment by the NTP was free of charge. In general, the subgroup had the same results as the main group (Table 2).The two outcomes of interest were referrals in the simulated patient method and the ability to identify suspected TB from the fictitious case on paper. The potential covariates of referring TB suspects to a TB hospital in the univariate analysis were sex, GPP certification and knowing that NTP treatment was free of charge. However, sex

did not show a significant correlation in the multivariate analysis. Work experience, being an assistant pharmacist and awareness about free NTP treatment were potential covariates for the detection of TB in the fictitious case in the univariate analysis, while only awareness about free treatment was a significant predictor in the multivariate analysis. Simulated patients had a higher probability of being referred directly to a TB facility if the HCP was aware about free NTP treatment (OR 5.80, 95%CI 1.88–19.62). Knowledge about free NTP treatment was also a predictor of the ability of HCPs to identify TB in the fictitious case (OR 5.14, 95%CI 2.36–11.73). GPP-certified pharmacies were less likely to refer the simulated patient to a TB facility than non-GPP pharmacies (OR 0.10, 95%CI 0.01–0.79) (Table 3).

DISCUSSIONS

Our study showed that about more than half of the HCPs were willing to dispense drugs either for the simulated patients or the fictitious case on paper. The drugs tentatively dispensed were mainly antibiotics, anti-inflammatory or anti-allergic agents and cough suppressants. Although 49% of the private pharmacies stored at least two first-line anti- tuberculosis drugs (data not shown), no anti-tuberculosis drugs were dispensed by self-prescription even when the simulated patient mentioned that he/she could have TB and asked for TB medication. The caution on the part of the private pharmacies in our study differed from the results from previous surveys in Ho Chi Minh City, where >20% of pharmacies were willing to dispense TB drugs without prescription (9,11). To some extent, this fiding reflects the success of the health authorities and the NTP in controlling indiscriminate dispensing of anti-tuberculosis drugs by private pharmacies. Nonetheless, the willingness to dispense non-TB drugs could increase the delay in TB diagnosis if the suspected patient was a real TB patient, and may explain why private pharmacies were potential sources of TB diagnostic delay in previous studies (15,16). Furthermore, patients who were referred to a general health facility could also be at risk of diagnostic delay, as the general health care services have also been reported to contribute to delay (15,16,18). Only approximately 10% of HCPs referred the simulated patient to the designated TB facility where diagnosis could be made without further delay.In this survey, 40% of the HCPs in private pharmacies were able to identify the fictitious case as a possible TB patient. This proportion is higher than the 18% reported in a previous study in Ho Chi Minh City, Viet Nam (2 test, $P < 0.01$) (11). A possible explanation is that we provided more TB-associated symptoms and not only those of 2 weeks' cough and fever.

Only about 30% of the HCPs knew that treatment given by the NTP was free, but those who did were fi ve times more likely to recognize TB and refer the patient to a TB facility. This suggests that HCPs who were aware about the NTP's free treatment policy performed better with regard to TB control. A possible explanation may be that these HCPs were willing to refer because they knew of the NTP policy, while the remaining HCPs, including those who claimed to know about the NTP, may not have known how to refer a TB suspect. Their decision was therefore drug prescription, referral to general hospitals or even suggestion to stay at home. The 30% awareness among HCPs about the free NTP treatment policy is lower than that reported in community-based surveys (19-21). Question four in the interview did not directly mention the free NTP treatment to avoid bias and that may be the reason for this difference. Our finding suggested that 70% of the HCPs in private pharmacies should be provided with more information about NTP policy in order to improve referral and prevent TB diagnosis delay.

A surprising result of the multivariate analysis is that GPP-certified pharmacies were less likely to refer simulated patients to TB facilities than non-GPP certified pharmacies (OR 0.10). Vietnamese guidelines for GPPs recommend referral of patients with community-acquired diseases to general health care.

Furthermore, the average work experience in the GPP group was 1.4 years less than that of the non-GPP group, although the difference was not statistically significant (*t*-test, $P = 0.17$), and their knowledge about NTP may be different. GPP pharmacies were therefore more likely to refer TB suspects to general health care. These findings suggest that GPP guidelines could provide more information on TB control and additional procedures for referral of TB suspects. The present study has a number of limitations. First, approximately half of the pharmacies randomly taken from the list could not be found, possibly because the private pharmacy network in Hanoi was changing rapidly at the time of the survey and the registration systems of health authorities had not yet been updated. Second, only two thirds of the private pharmacies that were found agreed to participate. The lack of cooperation by the other pharmacies may be due to the misunderstanding of the HCPs about the purpose of this research. The higher than expected number of pharmacies that remained unidentified or declined to participate may have affected the representativeness of our findings.

CONCLUSIONS

Less than half of the HCPs in private pharmacies could detect TB in a suspected patient, and a modest proportion of these referred a TB suspect to a designated TB facility where a diagnosis could be made without delay. Knowledge about free NTP treatment was a predictor for better TB case detection and referral; this could therefore shorten diagnostic and treatment delays.

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REFERENCES

(1) World Health Organization report 2009. Global tuberculosis control - epidemiology, strategy, finacing. 2009; Available at: http://whqlibdoc.who.int/publications/2009/9789241598866_eng.pdf. Accessed November, 2010.

(2) Hoa NB, Sy DN, Nhung NV, Tiemersma EW, Borgdorff MW, Cobelens FG. National survey of tuberculosis prevalence in Viet Nam 1. Bull World Health Organ 2010 04;88(1564-0604; 0042-9686; 4):273-280.

(3) Huong NT, Duong BD, Co NV, Quy HT, Tung LB, Bosman M, et al. Establishment and development of the National Tuberculosis Control Programme in Vietnam. Int J Tuberc Lung Dis 2005 Feb;9(2):151-156.

(4) Horie T, Lien LT, Tuan LA, Tuan PL, Sakurada S, Yanai H, et al. A survey of tuberculosis prevalence in Hanoi, Vietnam 1. Int J Tuberc Lung Dis 2007 05;11(1027-3719; 1027-3719; 5):562-566.

(5) Smith F. The quality of private pharmacy services in low and middle-income countries: a systematic review. Pharm World Sci 2009 Jun;31(3):351-361.

(6) Smith F. Private local pharmacies in low- and middle-income countries: a review of interventions to enhance their role in public health. Trop Med Int Health 2009 Mar;14(3):362-372.

(7) Lonnroth K, Thuong LM, Linh PD, Diwan VK. Utilization of private and public health-care providers for tuberculosis symptoms in Ho Chi Minh City, Vietnam. Health Policy Plan 2001 Mar;16(1):47-54.

(8) Lambert ML, Delgado R, Michaux G, Volz A, Van der Stuyft P. Tuberculosis control and the private health sector in Bolivia: a survey of pharmacies. Int J Tuberc Lung Dis 2004 Nov;8(11):1325-1329.

(9) Lonnroth K, Karlsson M, Lan NT, Buu TN, Dieu TT. Referring TB suspects from private pharmacies to the National Tuberculosis Programme: experiences from two districts in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 2003 Dec;7(12):1147-1153.

(10) Hurtig AK, Pande SB, Baral SC, Porter JD, Bam DS. Anti-tuberculosis treatment in private pharmacies, Kathmandu Valley, Nepal. Int J Tuberc Lung Dis 2000 Aug;4(8):730-736.

(11) Lonnroth K, Lambregts K, Nhien DT, Quy HT, Diwan VK. Private pharmacies and tuberculosis control: a survey of case detection skills and reported anti-tuberculosis drug dispensing in private pharmacies in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 2000 11;4(1027-3719; 11):1052-1059.

(12) Rajeswari R, Balasubramanian R, Bose MS, Sekar L, Rahman F. Private pharmacies in tuberculosis control--a neglected link. Int J Tuberc Lung Dis 2002 Feb;6(2):171-173.

(13) Uplekar M, Pathania V, Raviglione M. Private practitioners and public health: weak links in tuberculosis control. Lancet 2001 Sep 15;358(9285):912-916.

(14) Hoa NB, Cobelens FG, Sy DN, Nhung NV, Borgdorff MW, Tiemersma EW. Diagnosis and treatment of tuberculosis in the private sector, Vietnam. Emerg Infect Dis 2011 Mar;17(3):562-564.

(15) Lonnroth K, Thuong LM, Linh PD, Diwan VK. Delay and discontinuity--a survey of TB patients' search of a diagnosis in a diversified health care system. Int J Tuberc Lung Dis 1999 Nov;3(11):992-1000.

(16) Huong NT, Vree M, Duong BD, Khanh VT, Loan VT, Co NV, et al. Delays in the diagnosis and treatment of tuberculosis patients in Vietnam: a cross-sectional study. BMC Public Health 2007;7(1471-2458):110.

(17) FIRTH D. Bias Reduction of Maximum-Likelihood-Estimates Rid A-8207-2011. Biometrika 1993 MAR;80(1):27-38.

(18) Storla DG, Yimer S, Bjune GA. A systematic review of delay in the diagnosis and treatment of tuberculosis. BMC Public Health 2008 Jan 14;8:15.

(19) Lu SH, Tian BC, Kang XP, Zhang W, Meng XP, Zhang JB, et al. Public awareness of tuberculosis in China: a national survey of 69 253 subjects. Int J Tuberc Lung Dis 2009 Dec;13(12):1493-1499.

(20) Hoa NP, Chuc NT, Thorson A. Knowledge, attitudes, and practices about tuberculosis and choice of communication channels in a rural community in Vietnam. Health Policy 2009 Apr;90(1):8-12.

(21) Mushtaq MU, Majrooh MA, Ahmad W, Rizwan M, Luqman MQ, Aslam MJ, et al. Knowledge, attitudes and practices regarding tuberculosis in two districts of Punjab, Pakistan. Int J Tuberc Lung Dis 2010 Mar;14(3):303-310.

Table 1. Characteristics of the healthcare providers and private pharmacies

	Main group (n = 128)	Subgroup (n = 10)
Pharmacy		
Good pharmacy practice	30 (23)	1 (10)
Non-good pharmacy practice	98 (77)	9 (90)
Sex		
Male	20 (16)	1 (10)
Female	108 (84)	9 (90)
Age (years)	26 (23-37)	26 (23-39)
Degree		
Pharmacist	12 (9)	2 (20)
Assistant pharmacist	86 (67)	6 (60)
Drug seller	22 (17)	1 (10)
Training as assistant pharmacist	3 (2)	1 (10)
Medical doctor	1 (1)	0
Nurse	2 (2)	0
No answer	2 (2)	0
Work experience in private pharmacy (years)	4 (2-10)	4.5 (2-10)

Categorical variables are presented as number (%); continuous variables are presented as median (interquartile range)

Table 2. Response of the healthcare providers in the simulated patient method and the interview

	Main group (n = 128)		Subgroup (n = 10)
	n	Percentages (95% CI)	n (percentages)
Response to the simulated patients			
Sell drugs (#)	68	53 (44.9-61.3)	3 (30)
Antibiotics	53	41 (33.3-49.5)	2 (20)
Cough suppressants	30	23 (16.5-30.4)	3 (30)
Anti-inflammatory/allergy drugs	16	13 (7.1-17.9)	2 (20)
Others	12	9 (4.6-14.2)	0
Rest at home	1	1 (0.0-2.2)	0
Refer for diagnosis	59	46 (37.9-54.3)	7 (70)
Refer to General healthcare	48	38 (29.5-45.5)	4 (40)
Refer to TB healthcare	11	9 (4.0-13.2)	3 (30)
Response to the fictitious case			
Sell drugs (#)	58	45 (37.1-53.5)	2 (20)
Antibiotics	51	40 (31.8-47.9)	2 (20)
Cough suppressants	37	29 (21.4-36.4)	2 (20)
Anti-inflammatory/allergy drugs	31	24 (17.2-31.3)	1 (10)
Others	10	8 (3.4-12.2)	0
Refer for diagnosis	70	55 (46.5-62.9)	8 (80)
Possible diagnosis for fictitious case (#)			
Pneumonia	58	45 (37.1-53.5)	6 (60)
Tuberculosis	51	40 (31.4-48.3)	4 (40)
Bronchitis	50	39 (30.6-47.5)	3 (30)
Sore throat	7	5 (1.7-9.2)	0
Others	40	31 (23.6-38.9)	2 (20)
Aware of NTP			
Yes	103	80 (73.9-87.0)	10 (100)
No	25	20 (13.0-26.1)	0
Know s NTP's free treatment			
Yes	34	27 (19.3-33.8)	4 (40)
No	94	73 (66.2-80.7)	6 (60)

Others: all answers mentioned with frequency of less than 5%; (#): Multiple responses may occur;

Table 3. Predictors for the decision to refer and the ability to detect tuberculosis cases (n=138)(#)

	Refer simulated patient to TB hospital			Detect TB in paper fictitious case		
	n	(%)	OR (95%CI)	n	(%)	OR (95%CI)
Aware of free treatment in NPT						
No	5/100	(5)	1	29/100	(29)	1
Yes	9/38	(24)	5.80 (1.88-19.62)	26/38	(68)	5.14 (2.36-11.73)
GPP/non-GPP pharmacies						
Non-GPP	14/107	(13)	1			
GPP	0/31	(0)	0.10 (≤0.01-0.79)			

(#): Combination data from main group and subgroup was analysed

OR; odds ratio, adjusted for the other variables in the model (if present) by multivariate penalized logistic regression.

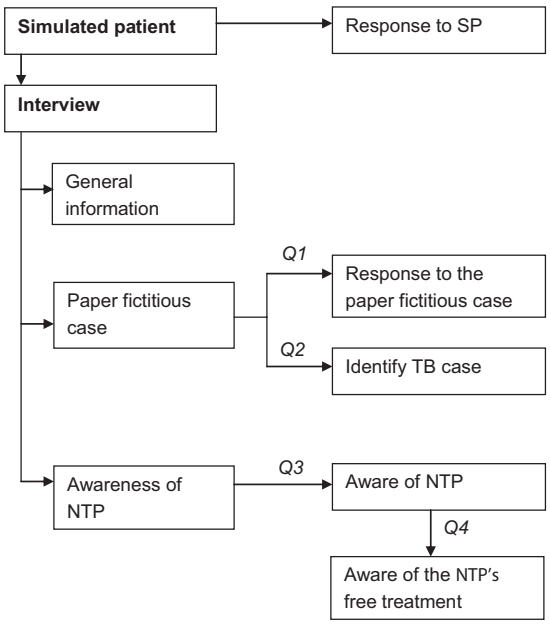

Figure. Study procedure

Chapter

3

Dispensing of anti-tuberculosis drugs in private pharmacies in Vietnam

Submitted

Dispensing of anti-tuberculosis drugs in private pharmacies in Vietnam
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ABSTRACT

Private pharmacies play an important role of a public-private mix strategy in Vietnam, but their role in storing and dispensing anti-tuberculosis drugs is not sufficiently understood. In this study on 138 private pharmacies we describe the availability of anti-tuberculosis drugs and dispensing practices. Anti-TB drugs presented more frequently in pharmacies located near two tuberculosis hospitals. Rifampicin was found in about 83% of private pharmacies, and about half were dispensed for indications other than tuberculosis. During interviews, 49 (35.5%) private pharmacies were willing to dispense fluoroquinolones for suspected tuberculosis patients. Eight pharmacies actually dispensed fluoroquinolones to simulated tuberculosis patients. Such improper dispensing of anti-TB drugs in private pharmacies deserves more attention.

INTRODUCTION

Vietnam is a high-burden tuberculosis (TB) country with an estimated incidence of 180,000 tuberculosis patients per year [1]. Hanoi is the second largest city in Vietnam, with a population of 3,000,000 and an estimated TB prevalence of 146 per 100,000 persons [2]. Previous studies conducted in Ho Chi Minh City, the largest Vietnamese city, showed that private pharmacies contributed substantially to the dispensing of anti-TB drugs [3]. The public-private mix (PPM) strategy, which is aimed at strengthening the cooperation between the National Tuberculosis control program and other private sectors, incorporated private pharmacies as a setting to refer TB suspected patients for diagnosis [4]. Whether private pharmacies could provide more in the PPM project remains an open question though. In Hanoi, previous studies addressed that self-prescription, especially with antimicrobial drugs, was very common in private pharmacies [5,6]. The consequence of this practice for TB control in Vietnam is currently unknown. However, we do know that fluoroquinolone-resistant strains have developed due to the empirical use of fluoroquinolones for indications other than TB [7]. Furthermore, the association between the Beijing genotype of *Mycobacterium tuberculosis* and higher fluoroquinolone resistance has been shown in a recent research in Vietnam [8]. As fluoroquinolones play a crucial role in the treatment of multidrug-resistant TB (MDR-TB) and fluoroquinolone resistance can develop extensively drug-resistant TB (XDR-TB), dispensing of these drugs deserves more attention and caution. Our studies aimed to explore the availability of anti-TB drugs in private pharmacies in Hanoi and the highlighted aspects in the dispensing of drugs to suspected TB patients.

POPULATION AND METHODS

The study consisted of a simulated patient method followed by an interview in private pharmacies in Hanoi, Vietnam. Thirteen pharmacies within a 500-meter radius from two TB hospitals in Hanoi (National Hospital for Lung Diseases and Hanoi Tuberculosis and Lung Disease Hospital) were collected to become a subgroup. From the remaining pharmacies, a sample of 125 was randomly taken as main group. The simulated TB patient (either a student or personnel of Hanoi University of Pharmacy) came to the pharmacy and complained about coughing and fever for four weeks with no improvement after two 10-day courses of antibiotics (amoxicillin followed by spiramycin). The simulated patient had contacted a TB patient, and suggested he could have TB. Then anti-TB drugs were requested. After that, an interviewer visited the private pharmacy and described a case: a 50-year-old male had suffered from coughing and fever for four weeks without improvement after a 10-day course of spiramycin. The interviewer asked what drugs may be dispensed for the described case. After the case detection, the interviewer asked what anti-TB drugs were available in the pharmacy with the help of a list with generic and brand names. Pharmacists who stored only rifampicin among first-line anti-TB drugs were asked about the dispensing purpose. The willingness to dispense fluoroquinolones for the described case was also explicitly questioned.

RESULTS

The study showed a high presence of first-line anti-TB drugs in the subgroup of private pharmacies, which were nearby the two TB hospitals. In other private pharmacies of the main group, first-line anti-TB drugs were less available, except for rifampicin. All pharmacies of the subgroup and 104 (83%) pharmacies of the main group had rifampicin in stock. Drugs for an MDR-TB indication were more frequently present in pharmacies near TB hospitals. These drugs, except for fluoroquinolones, were

uncommon in the main group and the mentioned purposes of dispensing were not TB. Fluoroquinolones were commonly available in private pharmacies. They were told to be dispensed for numerous indications but not for TB. Other anti-TB drugs (para-aminosalycilic acid, cycloserin, clofazimine and linezolid) were not available in private pharmacies (Table 1). Fifty-one pharmacies (37%) stored only rifampicin, whose most common purpose is to cover open wounds (84%).

Forty-nine (36%) interviewees claimed that they were willing to dispense fluoroquinolones for the suspected patient in the interview. Eight (5.8%) pharmacies sold fluoroquinolones to the simulated patient. The dispensed fluoroquinolones were ofloxacin, levofloxacin and gatifloxacin (Table 2). Most private pharmacy workers (136, 99%) were aware that prescriptions were required to dispense anti-TB drugs.

DISCUSSION

Our study showed different patterns of availability of anti-TB drugs stored in private pharmacies in Hanoi. Anti-TB drugs were more common in the private pharmacies near the two TB hospitals. This result suggests that the PPM program should encourage participation of these pharmacies in dispensing and monitoring the treatment of TB patients rather than referring suspected patients only [4]. The higher presence of rifampicin than other first-line anti-TB drugs suggests that it was there for purposes other than TB treatment. This was confirmed by the in-depth question: pharmacies solely stored rifampicin to dispense it for covering open wound infections. The risk of emerged drug-resistant TB from that practice might be low because it is used only locally. However, in a high TB-prevalence urban area like Hanoi, this risk is cannot be excluded [2].

No self-prescriptions containing TB drugs were initiated for simulated suspect patients and the case in the interviews. It is likely that the private pharmacies would not initiate treatment of TB if there were no confirmed diagnosis for it [3]. This was also confirmed by the high percentage (99%) of private pharmacists who were aware that a prescription is required for dispensing anti-TB drugs. Despite of this positive information, our results discovered that about 35% of pharmacies were willing to dispense fluoroquinolones to the case in the interview. It was not specified which fluoroquinolone they would be willing to dispense. However, in the simulated patient method the fluoroquinolones actually sold without prescription were ofloxacin, levofloxacin and gatifloxacin. According to the WHO guideline, these drugs play a crucial role in the treatment of MDR-TB [9]. Previous studies showed that empirical treatment with a fluoroquinolone not only delayed treatment for tuberculosis but also associated with a poor prognosis and developed fluoroquinolone-resistant *M. tuberculosis* strains [7,10]. Also, studies from Vietnam suggest that Beijing genotype of Mycobacterium tuberculosis, which has a higher potential of transmission, was significantly associated with high-level fluoroquinolone resistance [8]. To avoid the risk of emerged resistance to such back-bone drugs, we highlight the need to preclude the self-prescription of fluoroquinolones in private pharmacies, at least for patients with symptoms that highly suggest TB.

REFERENCES

[1] World Health Organization. Global tuberculosis report 2012. Geneva, Switzerland: WHO: World Health Organization; 2012.

[2] Horie T, Lien LT, Tuan LA, Tuan PL, Sakurada S, Yanai H, et al. A survey of tuberculosis prevalence in Hanoi, Vietnam. Int J Tuberc Lung Dis 2007 May;11(5):562-566.

[3] Lonnroth K, Lambregts K, Nhien DT, Quy HT, Diwan VK. Private pharmacies and tuberculosis control: a survey of case detection skills and reported anti-tuberculosis drug dispensing in private pharmacies in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 2000 11;4(1027-3719; 11):1052-1059.

[4] Quy HT, Lan NT, Lonnroth K, Buu TN, Dieu TT, Hai LT. Public-private mix for improved TB control in Ho Chi Minh City, Vietnam: an assessment of its impact on case detection. Int J Tuberc Lung Dis 2003 May;7(5):464-471.

[5] Chuc NT, Tomson G. "Doi moi" and private pharmacies: a case study on dispensing and financial issues in Hanoi, Vietnam. Eur J Clin Pharmacol 1999 Jun;55(4):325-332.

[6] Van Duong D, Binns CW, Van Le T. Availability of antibiotics as over-the-counter drugs in pharmacies: a threat to public health in Vietnam. Trop Med Int Health 1997 Dec;2(12):1133-1139.

[7] Chen TC, Lu PL, Lin CY, Lin WR, Chen YH. Fluoroquinolones are associated with delayed treatment and resistance in tuberculosis: a systematic review and meta-analysis. Int J Infect Dis 2011 Mar;15(3):e211-6.

[8] Duong DA, Nguyen TH, Nguyen TN, Dai VH, Dang TM, Vo SK, et al. Beijing genotype of Mycobacterium tuberculosis is significantly associated with high-level fluoroquinolone resistance in Vietnam. Antimicrob Agents Chemother 2009 Nov;53(11):4835-4839.

[9] WHO global Tuberculsois.Stop TB Dept. Treatment of tuberculosis guidelines [WHO/HTM/TB/2009.420]. : World Health Organization; 2010.

[10] Wang JY, Hsueh PR, Jan IS, Lee LN, Liaw YS, Yang PC, et al. Empirical treatment with a fluoroquinolone delays the treatment for tuberculosis and is associated with a poor prognosis in endemic areas. Thorax 2006 Oct;61(10):903-908.

Table 1. Availability of anti-TB drugs in private pharmacies

	Main group (n=125)			Subgroup (n=13)		
			For TB (*)			For TB(*)
	n	(%)	n	n	(%)	n
Rifampicin	104	(83)		13	(100)	
Isoniazid	36	(29)		11	(85)	
Streptomycin	39	(31)		12	(92)	
Pyrazinamid	23	(18)		11	(85)	
Ethambutol	33	(26)		11	(85)	
FDC	8	(6)		9	(69)	
Levofloxacin	55	(44)		12	(92)	
Gatifloxacin	32	(26)		5	(38)	
Moxifloxacin	13	(10)		3	(23)	
Ofloxacin	54	(43)		4	(31)	
Amikacin	14	(11)	0	12	(92)	8
Kanamycin	5	(4)	2	10	(77)	9
Capreomycin	1	(1)	0	0	(0)	0
Ethionamide	3	(2)	1	4	(31)	4
Prothionamide	1	(1)	1	3	(23)	3

(*): Mentioned to dispense for TB indication.

FDC: Fixed dose combination

Drugs that were not available: para-aminosalycilic acid, cycloserin, clofazimine, linezolid.

Table 2. Dispensing of fluoroquinolones during simulated patient method and reason for storing rifampicin (n=138)

	n	(%)
Willing to dispense fluoroquinolones for case in interview	49	(36)
Dispensed fluoroquinolones for simulated patient	8	(6)
Ofloxacin	4	(3)
Levofloxacin	2	(1)
Gatifloxacin	2	(1)
Stored only rifampicin	51	(37)
For cover open wound	43	(31)
For prescription	3	(2)
Others(*)	5	(4)
Knows that prescriptions are required for anti-TB drugs	136	(99)

FQs: fluoroquinolone; (*): frequency less than two

Part II.

Dried blood spot analysis for tuberculosis treatment optimization

Chapter

4

Dried Blood Spots: A New Tool for Tuberculosis Treatment Optimization

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Dried blood spots: a new tool for tuberculosis treatment optimization.
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ABSTRACT

Tuberculosis (TB) is a high-burden infectious disease, especially in low and middle-income countries. The efforts to eliminate this disease are challenged by the emergence of multi-drug resistance and TB-HIV coinfection. The cumulative knowledge on pharmacokinetics/pharmacodynamics of antituberculosis agents has recently encouraged therapeutic drug monitoring (TDM) in patient care. However, logistical problems related to conventional sampling limit the application of TDM in research-oriented institutions. Dried blood spot (DBS) compared with conventional venous blood sampling has the advantages of easier sampling, storage and transportation, thus enabling the application of TDM even in remote areas. In addition, DBS with its lower biohazardous risk can be safely performed in a high HIV prevalence area, which also tends to have a high TB burden. Another benefit of DBS sampling is that it requires a smaller blood volume than conventional sampling and is highly recommended for application in pediatric TB. A limitation of DBS is that additional considerations are required for analysis method development and validation. The accuracy of the DBS method is influenced by a number of factors that need to be thoroughly examined in method development and validation. Further, the agreement between DBS and plasma/serum concentrations is not always understood and further investigations are required.

INTRODUCTION

In 2008 there was an estimation of 11.1 million TB patients worldwide, totaling 9.3 million incident cases with a high mortality of about 1.3 million deaths (1). The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis and HIV/TB coinfection is also challenging the global target of the World Health Organization (WHO) of eliminating TB by 2050 (2). Treatments for such cases are affected by incredibly high costs and relatively low cure rates (3). Fortunately there is a growing understanding of the link between pharmacokinetics-pharmacodynamics (PK/PD) and the outcome of TB treatment. As a result, therapeutic drug monitoring (TDM) with drug dose adjustments based on PK/PD is applied more in TB control practice. However, these approaches have been challenged. The facilities required to perform PK/PD are advanced and are not available in all regular settings. The idea of establishing central, well-equipped laboratories where samples from remote clinical sites can be analyzed will make TDM more feasible in resource-limited countries. In this context, DBS sampling is more appropriate than conventional blood sampling for a number of reasons (4,5): (1) DBS sampling is easy to perform even without trained health care staff and requires no electrical facilities; (2) DBS samples can be stored and transported without cooling facilities because the drug is normally more stable in dried DBS matrix; (3) DBS with a minimal biohazardous risk is a good choice for high-HIV epidemic populations; (4) DBS requires small amounts of blood, making it suitable for pediatric TB; (5) DBS can be used for genotyping and facilitate large epidemiological pharmacogenetics studies.

Besides the advantages listed above, DBS also has drawbacks. The analysis of DBS samples normally requires more complex method development and validation process than conventional blood sampling. The influence of blood properties, sampling paper characteristic and method of extraction must be carefully examined. The agreement or correlation between analytical results from DBS and plasma, serum or whole blood samples are not always assured for all drugs, hence the clinical validation should be thoroughly investigated in order to properly interpret these results. Information about using DBS for antituberculosis drugs is still limited, therefore more research needs to be conducted in this area.

The present article will explore the options of DBS in TB treatment optimization, and underline considerations for developing and implementing a DBS sampling and analysis method for this purpose.

BENEFITS OF DRIED BLOOD SPOT SAMPLING FOR OPTIMIZATION OF TB TREATMENT

Therapeutic Drug Monitoring for TB Treatment

Short-course chemotherapy, which actually can take as long as 6 months or more, has been effectively used for newly diagnosed tuberculosis patients with a relapse rates of less than 5% in several clinical trials (6,7). The WHO has promoted "Directly Observed Therapy" (DOTS), which monitors patients' treatment adherence, is a cost-effective way to improve treatment outcome, and is especially recommended for patients at risk of non-compliance (8,9). However, something looks amiss with the recent treatment strategy because therapy outcomes were not reproducible for all DOTS programs in high-burden countries, where success rates widely vary from 56 to 94% (10). In addition, MDR-TB and XDR-TB will become a global threat if there are not enough efforts to prevent the spread of highly adapting *Mycobacterium tuberculosis* strains (11). While the number of available antituberculosis agents is limited and most agents have been launched for decades, several new drugs for the treatment of tuberculosis are still being researched (12,13). Hence we need to optimize the use of the available drugs

to improve treatment outcomes and prevent the *Mycobacteria* from further drug resistance. Recently, PK/PD studies have increased the knowledge about optimal treatment for tuberculosis (13-16). Consequently, application of TDM to tuberculosis treatment is encouraged because it provides a clearer scientific approach than the recent "treat and see" practice based on standardized drug regiments (13-17).

TB patients who have persistent symptoms and one positive bacteriological test after 2 months are potential candidates for TDM (16,17). This slow response may be a result of the quality of the drug, low adherence, malabsorption, drug interaction or the resistance of *Mycobacteria*. Measuring blood level of antituberculosis drugs can detect subtherapeutics and support the treatment decisions of clinicians. Low drug blood level result in ineffective pathogen inhibition and the risk of mutation and resistance (14,18,19). A study on TDM revealed that 46 and 48% of TB patients had low blood levels of rifampicin and isoniazid, respectively. Up to 83% of TDM service users thought it is valuable (20). Another TDM performed on patients with slow response to the treatment found that 52, 59 and 31% had low levels of rifampicin, isoniazid and ethambutol, respectively. After dose adjustment, 90% of patients with low rifampicin concentration achieved the expected level.

Diabetic TB patients should also be considered for TDM (17). Ray et al. suggested that a diabetic condition relates to higher risk of low rifampicin levels. The same conclusion was drawn in another study in which rifampicin blood levels in diabetic patients dramatically decreased. (21). However, the reason for the lower rifampicin exposure in diabetic patients is still being debated, as no noticeable difference was observed in yet another study (22). Because of these inconsistent results, monitoring antituberculosis drug concentrations can ensure the proper therapeutic outcome for diabetic TB patients.

MDR-TB patients can benefit from TDM because many second-line antituberculosis drugs have a narrow therapeutic window (23,24). Most recent MDR-TB treatment regiments are take 18 to 24 months of treatment with high risk of toxicity. TDM minimizes the toxicity related to high drug concentrations through adjustment of the dose. Low drug exposure, which can cause further resistance of MDR-TB, can also be detected.

The risk of developing active TB as an opportunistic infection is much higher in HIV patients, who can also benefit from TDM. The treatment of HIV/TB coinfection faces difficulties due to the high risks of drug-drug interaction. The hepatic cytochrome P450 (CYP) enzyme system plays an important role in the drug-drug interaction of antiretroviral and antituberculosis agents. The major metabolism of protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), which contributes to the backbone of highly active antiretroviral therapy (HAART), relies on CYP. Rifampicin, the crucial agent in TB treatment regimens, is also known as a potential CYP enzyme inducer and can cause subtherapeutic levels of PIs or NNRTIs if they are co-administered. As a result, the HIV is not controlled effectively and resistance to antiretrovirals also emerges. Indeed, avoidance of PIs is recommended in most cases treated with rifampicin due to the dramatically drop in blood levels. With NNRTIs, blood levels are less affected by rifampicin but an increased dose of NNRTIs is still required (25). Because this adjustment may have an adverse effect on the liver, TDM can be used to optimize the treatment. Rifabutin can be used as a substitute for rifampicin because of the smaller influence on CYP enzymes (26,27). However, the metabolism of rifabutin is inhibited by PIs and dose adjustment is still recommended (28,29). Besides drug-drug interactions, the condition of HIV disease itself can influence blood levels of antituberculosis (25,30-32). A retrospective cohort study showed that low serum levels of antituberculosis drugs are common among patients with advanced HIV. Subtherapeutic levels may

be due to malabsorption and can be overcome by increasing the dose. For these reasons, TDM is suggested as a potential tool to optimize therapy on this type of patients (32).

TDM is a useful tool for the optimization of TB treatment and improvement of treatment outcome. However, its application is still limited in health institutions and high-income countries. In low-income countries burdened by high TB rates, TDM seems to be a difficult option because of insufficient facilities and trained technicians. Centralizing facilities and workforces in one or two adequate laboratories is a possible solution. The difficulty however lies in how to integrate different clinical sites with the analytical laboratory. TDM has recently been using liquid samples, including serum, plasma and whole blood as conventional matrices. Venous blood sampling normally requires trained health care staff, large blood amounts, complex cooling facilities for storing and transporting samples, and careful prevention of biohazards. DBS that is performed well in a limited setting can minimize such logistical difficulties (33). DBS has been used for decades to screen biomarkers and monitor treatment outcomes of HIV. Thanks to recent precise analytical instruments (e.g. LC-MS/MS), DBS is more widely applied in pharmacokinetic studies and TDM (4,34-36). DBS can be attained by spotting small blood drops from pricking an adult's finger or an infant's heel onto a pure cellulose paper strip (Figure 1). The drug concentration is then analyzed after appropriate extraction.

DBS and Therapeutic Drug Monitoring in Resource-limited Settings

The most attractive aspect of applying DBS sampling is that it can be performed even in limited resource areas. First, DBS sampling is quite easy and even patients themselves can perform it after a simple instruction by a health care personnel (33). The convenience of DBS sampling is important because TB patients without XDR-TB are mostly treated in the community under the DOTS program, where trained nurses are not always available to take blood samples. Some studies have been conducted to explore the possibility of patients themselves doing DBS sampling and whether those DBS results are reliable. Data from transplant patients using immunosuppressants and diabetic patients show that there is no difference from DBS obtained by nurses or by self-sampling at home (37). In another study a slight difference was detected when patients took the sample themselves at home instead of under supervision at a clinic (38). In terms of tuberculosis control, we noticed that the DOTS program is spreading globally, even in rural areas. The person who directly observes the therapy of the patient in this program can be trained to take the samples and handle them if the TDM is performed in such situations.

Second, DBS sampling does not require electrical apparatuses for centrifuging or a volumetric device, therefore it is considered to be highly suitable for a resource-limited setting. Indeed, DBS is recommended for monitoring HIV treatment or screening biomedically in anthropology surveillance resource-limited settings to overcome the logistical burden of conventional sampling (4,5). These successful applications encourage us to think about DBS when designing a population-based pharmacokinetic study of tuberculosis agents or setting up a TDM program in the remote area with a high TB burden.

Third, differently from serum or plasma which are available as liquid matrices, DBS is stored as a dried sample, therefore improving stability (33,36). As a consequence, the DBS sample is easier to handle because dry ice for shipment and a freezer for storage is not required. For example, TDM for rifampicin and isoniazid has required the plasma sample to be stored immediately at 70°C and transported with dry ice to the laboratory within four days of collection (20). Another TDM procedure for antitubercu-

losis agents reported that serum was immediately placed in a 70°C freezer and then packed in dry ice and shipped to the research centre. The results of the TDM were faxed and mailed back to the originating chest clinic (39). Obviously, these procedures are not applicable in a resource-limited setting and a validated DBS method with an appropriate stability assessment can be used as an alternative.

Therapeutic Drug Monitoring of HIV/TB Coinfection

The application of DBS has been widely investigated in high HIV prevalence. DBS has been recently used as an ideal tool to monitor viral load or even generate genotype of the HIV-resistant strain for patient care in a resource-limited setting (4,40). Integrating with HIV monitoring programs using DBS, additional benefit in patient care can be expected. For that reason, DBS should be further investigated for TDM or pharmacokinetics research on such patients. (40). Most TDM trials on antiretrovirals have been conducted in high-income countries and did not show a clear benefit. It is suggested that research be conducted in more appropriate places where the burden of disease is greatest and the concurrent epidemics (tuberculosis, HIV, malaria) are available (41). High HIV/TB burden areas are commonly resource-limited and the application of DBS for such research should be considered (10). Besides the advantages mentioned above, using DBS sampling can minimize the risk of biohazard infection. Because it is a dried sample no tube is needed, and the risk of contamination from broken samples can be avoided. In addition, viruses like HIV lose their infectivity in dried paper matrix and the DBS sample can be packed and transported by regular mail without concerns of handlers being exposed to the blood or infectious material (4,33,42).

DBS and TB Treatment Ooptimization in Pediatrics

Study on pharmacokinetics of antituberculosis drugs in term of infants and children is still required (43,44). In the case of tuberculosis treatment, the dosage for children was previously defined extrapolating pharmacokinetics information from adults. The most recent revision of first-line drugs using pediatric pharmacokinetic data suggests higher doses than previous recommendations in order to get a blood level equal to that of adults' (45). However, pharmacokinetics data to confirm the appropriateness of these new updated dosages is not available and the pharmacokinetics information on other antituberculosis drugs is still limited. The WHO recently called for a large population-based pharmacokinetic study at the newly recommended dosages and pharmacokinetic studies in infants (43). Using large amounts of blood is the ethical barrier currently faced by pharmacokinetic studies on pediatrics (46,47). To complete a pharmacokinetic profile, about 10 samples are required. Population pharmacokinetics using population-based estimation is a possibility for surmounting this obstacle. Because information from all individuals is merged when estimating population pharmacokinetic parameters, the sparse sampling scheme, which requires only one to six time points, can be applied. For pharmacokinetic studies on pediatrics, this small number of samples is preferable to the traditional intensive sampling scheme which normally requires samples at more than ten time points (47,48). However, even with a sparse sampling scheme, the 3 ml of blood per sample required in conventional sampling seems too much for children (46). DBS, which requires only about less than 100 μL of blood, can be considered as an alternative sampling method. The application of DBS has a long-standing history since the first collected DBS sample from newborn heel pricks to detect phenylketonuria in the 1960s (49). This led to several nationwide newborn screening programs for metabolic disease and is now applied worldwide (50-52). DBS was also successfully applied in pharmacokinetic studies of antimicrobial drugs on neonates (53). Because it requires small amounts of blood, DBS should be used in pharmacokinetic studies or TDM for children (46).

Pharmacogenetics for Pharmacovigilance

DBS samples can be used for genotyping purposes, thus enabling performance of pharmacogenetics. The recently clinically validated pharmacogenetic biomarker related to metabolism of rifampicin and isoniazid is N-acetyltransferase. In combination with TDM, this biomarker can be a complementary tool for optimal individualization therapy (54). De Boer et al. used DBS for genotyping of CYP450 enzymes from 12 volunteers, observing a 100% match result between DBS and whole blood drawn by venipuncture (55). This promising finding, in conjunction with the possibility to apply DBS in remote areas, suggest DBS as a potential sampling method for the future application of pharmacogenetics to tuberculosis treatment.

DEVELOPMENT AND VALIDATION OF A DRIED BLOOD SPOT METHOD

DBS has many advantages but also some disadvantages. Several considerations are required to ensure accuracy when DBS methods are developed and validated. Similarly to other bioanalytical methods, DBS methods need to be validated in term of selectivity, specificity, linearity, reproducibility, recovery and stability. Currently the most accepted guideline for the validation of bioanalytical methods is the "Guidance for Industry. Bioanalytical Method Validation" of the U.S. Food and Drug Administration (56). DBS analytical results can also be affected by a list of confounding factors, hence extra assessments in the method development and validation need to be considered (33,36).

Sampling Paper

There are several paper brands with different characteristics that can be used in DBS sampling. Schleicher & Scheull 903 (Whatman 903) has been widely applied in newborn screening and HIV monitoring programs for years (4,52). In genotype HIV studies there was evidence that Whatman FTA Elute paper was inferior to Whatman 903 (4). Another paper, Whatman 31 ET-CHR, which is thicker than the other two types, was also applicable for a DBS method (57). Regardless of the type of paper used, paper types are not interchangeable because the difference in thickness and porosity leads to different absorption properties. For that reason, the paper used in DBS sampling needs to match the DBS calibration in the laboratory. The uniformity of paper from different batches should also be tested. The Newborn Screening Quality Assurance Program applies a procedure in which ^{125}ILThyroxine is used to test the homogeneity from each batch (52). A simpler way to estimate the spread of blood on the paper is by measuring the size of DBS produced by different blood volumes. The linear correlation between the area of DBS and the blood volume can indicate whether the blood spreads equally on the paper. (57-59). In some situations the paper can also be treated with an appropriate reagent before it is used for sampling in order to improve stability or recovery of the DBS (36,60). However, the consistency of any additive needs to be proved.

Sampling Method and Extraction

There are two approaches for DBS sampling. The first method (method 1) uses a precise volumetric device (e.g. pipette) to withdraw capillary blood from a finger prick and transfer to paper in order to make the blood spot. The whole DBS is then cut or punched out for analysis (61-64). Because the blood volume has already been precisely defined in each blood spot, errors caused by the non-uniformity of the paper, hematocrit, chromatographic effects or blood volume of the spot can be neglected. In the second method (method 2), the blood volume in a DBS does not need to be precise because a fixed diameter disc from a part of the DBS is punched out for analysis. In most cases, the DBS is produced by directly dropping blood from a finger prick onto the paper (57,58,65-67). This approach is more con-

venient for DBS sampling in a resource-limited setting where volumetric devices are not available or cannot be easily handled by untrained personnel. The drawback of this technique is that the effect of blood volume variation on the accuracy of the assay needs to be further examined. An improved way to generate DBS is by withdrawing blood from a finger prick into an anticoagulant-treated capillary and subsequently spotting on the paper. Using the capillary can help the technician control the blood volume better than directly dropping blood from a finger prick. Despite the improved work flow and the more consistent blood volume in the DBS, using a capillary might be not very convenient in settings with limited resources. Because a fixed diameter disc from one part of the DBS is used for analysis, this approach should be similar to method 2 (55).

Recent DBS sampling as described above applies mostly to off-line extraction, which is time-consuming and labor-intensive, and sometimes requires many manual procedures (36). For that reason, on-line extraction has been introduced as an effort to improve the throughput of the DBS analysis. The first introduced on-line elution system integrated an extraction cell with an extraction column for purification and an LC-MS/MS system (68,69). This on-line extraction however still required a manual step to punch the disc out and place it in the extraction cell. The other concept is to directly elute one part of the DBS with the help of a clamp module or a thin-layered chromatography mass spectrometer interface (TLC-MS) (70,71). These instruments do not require the punching step. The integration with an auto-sampler system using a standardized DBS card enables the totally automatic analysis (35). Nevertheless, it should be noticed that these on-line extraction instruments are still being developed. Improvement and more data are needed to prove their validity in practice. The DBS extraction procedures are presented in Figure 2.

Blood Properties

Beside the convenience of DBS sampling in a resource-limited setting, the drawback of method 2 is the occurrence of analytical biases caused by hematocrit, blood spot volume and chromatographic effect (33,36). Hence these factors should be considered in method validation. The accepted biases should be within the criteria in bioanalysis of 15% (56). These biases can be neglected if the DBS analysis follows method 1, as the entire DBS is used for analysis.

Hematocrit

The hematocrit of patients varies between individuals. Because hematocrit accounts for the proportion of blood cells in the whole blood, it contributes to its viscosity. With higher hematocrit the spread of blood is limited, resulting in a smaller DBS than with lower hematocrit. (57,59). Because a fixed diameter disc is punched out, the actual blood amount in a disc with higher hematocrit is larger too. The analytical biases between different hematocrit blood could thus be observed (57,59,72,73). For that reason, the hematocrit of calibration DBS samples should be standardized at a specific value (33). The hematocrit of a tuberculosis patient is approximately 35 ± 6%, a little bit lower than a healthy person's; a standardized hematocrit value of 35% can thus be selected (74,75). A validation using quality control samples with hematocrit ranges from 20 to 50% (e.g. ± 2 standard deviations) should show an acceptable bias of less than 15% for quantitative bioanalysis (56). The blood with hematocrit largely differs from standardized value, can produce a high bias, and the correction for hematocrit may improve the accuracy of the method. Vu et al. developed an equation for the correction of analytical results for hematocrit. Normally, hematocrit is part of routine laboratory tests for patients with tuberculosis and can be used for this correction (57). However, the acceptability and the application of this correction method in clinical practice should be further investigated and validated

To prepare calibration and quality-control samples for DBS analysis, blood with a defined hematocrit value is required. This blood can be prepared by removing or adding appropriate volumes of plasma from the blood (59,76). Another method is washing the red blood cells by physiological buffer and subsequently mixing with plasma (57,77). It must be noticed that the reverse pipette technique should be used to transfer a precise volume of a high-viscosity suspension like whole blood or red blood cells. This technique can reduce errors due to film retention.

Chromatographic Effect

The diffusion of blood over the paper may be non-homogenous. Holub et al. found that several amino acids have different concentrations between the central and the peripheral parts of the DBS. To test this effect, analytical results from 3-mm discs punched out from the central and the peripheral parts of the DBS were compared (78). Another approach used different punch sizes, verifying if the concentrations show a linear correlation with the areas of the punched discs (66). As long as the analytical biases of different punches to nominal concentrations are less than 15%, the chromatographic effect can be neglected.

Blood Volume of the Spot

A punch diameter between 3 and 8 mm is commonly used for the DBS analysis following method 2 (33,36). It is important that the punch disc be small enough to be fully filled by blood and large enough to ensure acceptable assay sensitivity. In a resource-limited setting where no volumetric capillary device is available, the blood spot size may tend to vary and analytical bias may be introduced as a consequence (79). The biases caused by the variation in blood spot volumes can be partly explained by the chromatographic effect because the disc punched out from smaller DBS will contain more peripheral parts (57). If the drug is more concentrated in the central part, the smaller DBS seems to introduce negative bias. However, this effect is not very common with recent developed DBS methods (57,79).

Matrix Effect and Recovery

The common instruments used for DBS analysis are immunoassay, gas or liquid chromatography integrated with UV detectors, and tandem mass spectrometry. One has to keep in mind that cellular components (e.g. protein, hemoglobin, Fe^{2+}, etc.) present in DBS can produce significant matrix effects. Takashi et al. observed a significant bias due to hemoglobin when a DBS method for gentamicin using immunoassay was developed (76). With mass spectrometry, the matrix effect is an even more complex aspect. In this case, either ion suppression or enhancement may happen and this needs to be examined in method validation (36,56,80). If the reproducibility of the method is potentially affected by the matrix effect, liquid-liquid extraction (LLE) or solid phase extraction (SPE) can be applied. LLE or SPE can effectively eliminate the endogenous component in DBS, therefore improving the reproducibility of the method (81-83). Because the analyte of interest needs to be extracted from the DBS, both in the on-line or off-line extractions, the recovery of the extraction needs to be evaluated. The validated procedure in off-line extractions should be carefully complied with by the analysts because non-standardized performance may introduce assay errors. In our laboratory we experienced that DBS can be gradually extracted without any stimulation like sonication or vortex. If the DBS is not discarded after extraction or the extracts are withdrawn at different times, the recovery may be not reproducible. A summary of how to evaluate the matrix effect and recovery in a DBS method can be found in another review (36).

Stability in Relation Application in Remote Areas

As mentioned before, one of the most attractive aspects of DBS sampling is that it can overcome logistical problems, which is challenging conventional sampling methods. For that purpose, it is expected to store and transport DBS at normal temperatures without dry ice or freezer. Nevertheless, using DBS is not the solution for all problems. First, some molecules are not stable in the dried matrix of DBS even at low temperatures (84). Second, regular shipping sometimes introduces extreme environmental conditions, including high temperature and high humidity. Such conditions can stimulate the degradation of DBS (33,36). For that reason, the stability of any DBS method needs to be thoroughly validated under all possible conditions happening during storage and transportation.

Effect of Temperature

Temperature is the most noticeable factor that needs to be investigated and carefully controlled, otherwise it may limit the application of DBS to a certain extent. Some antituberculosis agents such as isoniazid are not stable in plasma even at 20°C (85). In such situations, the conventional sample is difficult to handle, even in well-equipped settings. Using DBS may help the samples to become more stable. Several problems remain though, as some substances are not stable in DBS even at cool temperatures of 4°C and hydrolysis occurs even during DBS drying time (84). For that reason, examination the stability of the whole blood during drying time of the DBS should be performed. The drying time depend on the amount of blood, paper type and the environment condition like humidity, temperature (33,36).

The stability can still be improved by adding inhibitors. The antimalaria agents amodiaquine and lumefantrine are not stable in either plasma or DBS at room temperature (83,86). Consequently, application to the field setting was limited. Ntale et al. introduced an improvement in which phosphoric acid was added to the blood before producing a DBS in order to prevent the oxidation. With this adjustment, lumefantrine was stable for 3 months and amodiaquine was stable for at least 4 months at the room temperature of 25°C. In this way, sampling of these antimalaria agents in the field becomes more feasible (61,62).

Many high-burden tuberculosis countries and resource-limited settings have tropical environments with high temperatures. This should be taken into account when validating for stability before applying the method. For TDM in remote areas, DBS samples are expected to be transported by mail. As temperatures in a postal box can reach 60°C, this condition should be tested too (33). The duration of the stability test needs to be defined based on real conditions. Extreme temperatures up to 60°C may not present for the whole duration of the sample shipment, therefore a short term of 3 days of stability is considered appropriate (60). Another way to examine stability conditions is to send DBS samples by post and measure the subsequent degradation (37,87). Recently, battery powered temperature and humidity monitors are applied in the transportation of plasma sample. This device can be used to examine the condition of DBS shipment. The information about shipment temperature and humidity is useful to figure out which conditions should be test in the validation of stability.

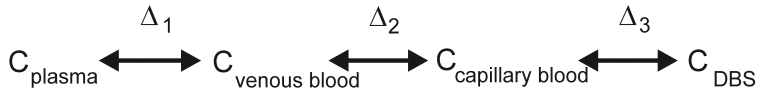
Effect of the Humidity

Another noticeable factor that needs to be controlled is humidity. In some field settings humidity is very high, even reaching saturation. Humidity can activate hydrolysis or enable the growth of microorganisms (36). In addition, high humidity requires longer drying time, which can induce degradation. Using heat or light for drying acceleration can be a solution but not for thermo-unstable agents (88). Humidity should be avoided by storing in waterproof plastic bags with desiccant sachets. A humidity indicator should be added to the bag also (33,36).

APPLICATION OF DBS FOR CLINICAL USE

Relationship between Concentrations from DBS and Conventional Method

As mentioned, introducing DBS to the optimization of tuberculosis treatment is attractive and can be considered as a promising tool in patient care. With certain drugs such as immunosuppressants and antimalaria agents, the pharmacokinetic studies or TDM were based on venous whole blood concentrations, which are supposed not to be very different from DBS concentrations (37,89-92). However, for other drugs, the recent available pharmacokinetics data is based on plasma or serum concentrations. As a consequence, dose optimization should also use the conventional sampling method. To apply DBS in that case, one should consider the possible differences between the concentrations in DBS and in plasma/serum. Besides the random error due to analytical instrument error, a number of potential technical and physiological confounders may result in biases among drug concentrations in venous plasma (C_{plasma}), venous blood ($C_{venous\ blood}$), capillary blood ($C_{capillary\ blood}$) and DBS (C_{DBS}).



The bias between C_{plasma} and $C_{venous\ blood}$ (D_1) is introduced due to the different extents of the partition between erythrocytes and plasma. In addition, individual hematocrit variations also result in this kind of bias. Normally, the blood/plasma ratio (B/P) depends on hematocrit while the erythrocyte/plasma ratio (E/P) is independent from it (93,94). It should be remarked however that when plasma protein binding and/or erythrocyte binding sites are saturated, the nonlinear kinetics of the erythrocyte partition appear. As a consequence, in that case the E/P and B/P will not be constant over the investigated concentration range (95). The available data on the influence of pathological and demographic factors on the B/P are limited. Disease, gender, age, genetics and co-medication may be factors that also contribute to the variation of B/P (93,95).

Besides the extent of B/P partitioning, the rate of partition may also cause disagreement between C_{plasma} and $C_{venous\ blood}$. Partitioning equilibrium is rapidly achieved for most of the drugs, therefore it can be neglected. However, some drugs require hours to reach the equilibrium, so the agreement between blood and plasma concentrations will not be warranted (96). Drugs with high binding affinity for plasma proteins or erythrocytes and with low cell membrane permeability are potential candidates for partitioning delay (95). Information about the partitioning of drugs between blood and plasma is limited so far. This information is important for the development of DBS method because it allows researchers to predict whether a method is applicable (97). A small in vitro experiment can help roughly estimate this factor.

The bias between $C_{venous\ blood}$ and $C_{capillar\ blood}$ (D_2) may also be a source of error. The capillary blood from a finger prick may be contaminated by interstitial or intracellular fluid. It has been therefore suggested that the first drop of blood be discarded. Pressing the finger to collect more blood should also be avoided because it may introduce more contaminating fluid. Warming the finger with warm water can facilitate the blood flow and enable easier DBS sampling (33). However, the warm water may also change the circulation of capillary blood and its properties as a consequence and should be examined too. Capillary blood is a mixture of arteriolar and venous blood, and comparable results between two sampling sites are not always expected. Spooner suggested that the concentration for capillary blood

should be between the concentration of arterial and venous blood. The distribution of some drugs among extravascular tissue, arterial and venous system may be delayed and cause the difference of venous and capillary blood. (46,93,98). It was reported that concentrations of paracetamol in finger-prick DBS were significantly different than those in venous DBS taken at the same time. (98,99). However, no significant differences were observed for other drugs like tacrolimus, everolimus clozapin or moxifloxacin (37,57,60,100).

Factors that influence the difference between $C_{capillary\ blood}$ and C_{DBS} (D_3) are mainly introduced by the sampling technique. The hygienic solvent should be dried at the puncture site to avoid hemolysis or sample contamination (33). Viscosity, paper type and chromatographic effect, which were mentioned above, may also contribute to this kind of error.

Clinical Validation

Because many factors can potentially influence the difference between the DBS and plasma concentrations, it is recommended that a DBS method be validated on actual patients (33,36). The purpose of clinical validation is to demonstrate the agreement or the correlation between the DBS and plasma concentrations. With a sufficient correlation, appropriate interpretation of DBS results to plasma or whole blood is possible and can be used for clinical decisions. In clinical validation, the drug concentration in DBS and other conventional samples taken at the same time are measured and compared by using a linear regression analysis. A high correlation coefficient suggests that the results between methods can be interchangeable (101). However, to explore the agreement of different methods, the comparison method using Deming regression or Pasing-Bablok regression is preferable (90,102). These kinds of regressions take into account the errors from both test and reference analytical methods instead of the error from only test methods used in ordinary linear regression (103). The slope of the regression line is compared to 1 and the intercept is compared to zero. The slope is different from 1 if DBS and plasma concentrations are different. The uneven plasma-blood cell partitioning is a potential reason for this kind of difference. Regardless of the regression method used, a nonlinear regression can be observed if there is a nonlinear partitioning kinetic in which E/P ratio is not constant over the investigated concentration range. In that case, the interpretation from DBS concentration to plasma results seems to be complex. Another way to evaluate the agreement between two concentrations is using Bland Altman plot. In this method, the differences between two concentrations are plotted against the average concentrations. The average of the differences was compared to zero to show the bias, and the 95% limit of agreement presents the variation between two concentrations (90,98,100,101). Another, more clinical approach is to evaluate the agreement of pharmacokinetics information attained from DBS and venous blood concentrations. Cheung et al. used Bland Altman plot to compare the area under the concentration time curves of tacrolimus constructed by DBS and whole blood results (89). The bias of 8.3% between the result from the two samples is considered to be acceptable, therefore the DBS result can be used to estimated tacrolimus concentration in the whole blood.

DRIED BLOOD SPOT METHOD FOR ANTITUBERCULOSIS AGENTS

Up till now, there has been a limited number of publications about the DBS method for antituberculosis agents. Allanson et al. have developed a method to determine rifampicin in DBS by using HPLC with UV detection. Rifampicin from DBS was extracted using acetonitrile and ammonium acetate buffer. There was no degradation, neither in the DBS nor in the post-preparation solution within 9 hours (67). The same research group also put their efforts into developing a method to determine isoniazid

and pyrazinamide in DBS based on gas chromatography-mass spectrometry. The primary result was promising with pyrazinamide but the oxidation was observed with isoniazid. The instability of isoniazid should be noticed when developing and validating a DBS method for this drug (104). Another publication found is about determination of moxifloxacin, an antituberculosis agent for MDR-TB, in DBS using LC-MS/MS analysis. The method was validated and can be used in future pharmacokinetics studies or TDM (57).

The correlation between DBS and plasma concentrations of rifampicin has been tested in five patients and shown to be linear. The slope of 0.72 suggests that the concentration in DBS is lower than in plasma, and that more rifampicin distributes in plasma (67). With another antituberculosis agent, moxifloxacin, the slope of Passing Bablok regression line was 1.49 (90%CI:1.32-1.77, n=18). This reveals that moxifloxacin has a higher affinity for blood cells than for plasma (57). Correction for these distribution factors is required to interpret the DBS results to plasma concentrations for clinical purposes. Future development of more DBS methods for the other antituberculosis agents and applying DBS to TB treatment optimization may prove the further validity of this method.

CONCLUSION

Application of dried blood spots sampling for the optimization of tuberculosis treatment might be the solution for overcoming the obstacle of conventional sampling. In comparison to conventional sampling methods, DBS sampling is easier to perform in a resource-limited setting and easier to transport to the laboratory. These advantages enable TDM even in rural areas with an existing demand for this service. In addition, DBS sampling is preferable for TDM of TB drugs in pediatric patients because it requires small amounts of blood, which is more acceptable than in conventional sampling. However, additional considerations are required when applying this method in daily practice. The accuracy of the method may be affected by many factors, including paper type, blood characteristics, analysis method and the characteristic of drugs themselves. Clinical validation of this method is required for the interpretation of DBS concentrations into serum or plasma concentrations. In the future, more DBS methods for antituberculosis agents should be developed and applied to patient care.

REFERENCES

(1) World Health Organization. Global tuberculosis control: a short update to the 2009 report. 2009; Available at: http://whqlibdoc.who.int/publications/2009/9789241598866_eng.pdf. Accessed January, 2011.

(2) Onozaki I, Raviglione M. Stopping tuberculosis in the 21st century: goals and strategies. Respirology 2010 Jan;15(1):32-43.

(3) Suarez PG, Floyd K, Portocarrero J, Alarcon E, Rapiti E, Ramos G, et al. Feasibility and cost-effectiveness of standardised second-line drug treatment for chronic tuberculosis patients: a national cohort study in Peru. Lancet 2002 Jun 8;359(9322):1980-1989.

(4) Johannessen A. Dried blood spots in HIV monitoring: applications in resource-limited settings. Bioanalysis 2010 Nov;2(11):1893-1908.

(5) Benyshek DC. Use of dried blood spots: an ideal tool for medical anthropology "in the field". J Diabetes Sci Technol 2010 Mar 1;4(2):255-257.

(6) Hong Kong Chest Service/British Medical Research Council. Five-year follow-up of a controlled trial of five 6-month regimens of chemotherapy for pulmonary tuberculosis. Am Rev Respir Dis 1987 Dec;136(6):1339-1342.

(7) British Thoracic Society. A controlled trial of 6 months' chemotherapy in pulmonary tuberculosis. Final report: results during the 36 months after the end of chemotherapy and beyond. Br J Dis Chest 1984 Oct;78(4):330-336.

(8) Combs DL, O'Brien RJ, Geiter LJ. USPHS Tuberculosis Short-Course Chemotherapy Trial 21: effectiveness, toxicity, and acceptability. The report of final results. Ann Intern Med 1990 Mar 15;112(6):397-406.

(9) Wilkinson D, Davies GR, Connolly C. Directly observed therapy for tuberculosis in rural South Africa, 1991 through 1994. Am J Public Health 1996 08;86(0090-0036; 8):1094-1097.

(10) World Health Organization report 2009. Global tuberculosis control - epidemilogy, strategy, finacing. 2009; Available at: http://whqlibdoc.who.int/publications/2009/9789241598866_eng.pdf. Accessed November, 2010.

(11) Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, van Soolingen D, et al. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet 2010 MAY 22;375(9728):1830-1843.

(12) Mitnick CD, McGee B, Peloquin CA. Tuberculosis pharmacotherapy: strategies to optimize patient care. Expert Opin Pharmacother 2009 FEB;10(3):381-401.

(13) Dartois V, Barry CE. Clinical Pharmacology and Lesion Penetrating Properties of Second- and Third-Line Antituberculous Agents used in the Management of Multidrug-Resistant (MDR) and Extensively-Drug Resistant (XDR) Tuberculosis. Curr Clin Pharmacol 2010 02/16(1574-8847).

(14) Pasipanodya J, Gumbo T. An Oracle: Antituberculosis Pharmacokinetics-Pharmacodynamics, Clinical Correlation, and Clinical Trial Simulations To Predict the Future. Antimicrob Agents Chemother 2011 JAN;55(1):24-34.

(15) Davies GR, Nuermberger EL. Pharmacokinetics and pharmacodynamics in the development of anti-tuberculosis drugs. Tuberculosis (Edinb) 2008 Aug;88 Suppl 1:S65-74.

(16) Nuermberger E, Grosset J. Pharmacokinetic and pharmacodynamic issues in the treatment of mycobacterial infections. European Journal of Clinical Microbiology & Infectious Diseases 2004 APR;23(4):243-255.

(17) Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis. Drugs 2002;62(0012-6667; 15):2169-2183.

(18) Unsalan S, Sancar M, Bekce B, Clark PM, Karagoz T, Izzettin FV, et al. Therapeutic monitoring of isoniazid, pyrazinamide and rifampicin in tuberculosis patients using LC. Chromatographia 2005 JUN;61(11-12):595-598.

(19) Rodriguez JC, Cebrian L, Ruiz M, Lopez M, Royo G. Mutant prevention concentration of isoniazid, rifampicin and rifabutin against Mycobacterium tuberculosis. Chemotherapy 2005 May;51(2-3):76-79.

(20) Ray J, Gardiner I, Marriott D. Managing antituberculosis drug therapy by therapeutic drug monitoring of rifampicin and isoniazid. Intern Med J 2003 05;33(1444-0903; 5-6):229-234.

(21) Nijland HMJ, Ruslami R, Stalenhoef JE, Nelwan EJ, Alisjahbana B, Nelwan RHH, et al. Exposure to rifampicin is strongly reduced in patients with tuberculosis and type 2 diabetes. Clinical Infectious Diseases 2006 OCT 1;43(7):848-854.

(22) Ruslami R, Nijland HMJ, Adhiarta IGN, Kariadi HKS, Alisjahbana B, Aarnoutse RE, et al. Pharmacokinetics of Antituberculosis Drugs in Pulmonary Tuberculosis Patients with Type 2 Diabetes. Antimicrob Agents Chemother 2010 MAR;54(3):1068-1074.

(23) Douglas JG, McLeod MJ. Pharmacokinetic factors in the modern drug treatment of tuberculosis. Clin Pharmacokinet 1999 AUG;37(2):127-146.

(24) Yew WW. Therapeutic drug monitoring in antituberculosis chemotherapy. Ther Drug Monit 1998 10;20(5):469-472.

(25) Elsherbiny D, Cohen K, Jansson B, Smith P, McIlleron H, Simonsson US. Population pharmacokinetics of nevirapine in combination with rifampicin-based short course chemotherapy in HIV- and tuberculosis-infected South African patients. Eur J Clin Pharmacol 2009 Jan;65(1):71-80.

(26) Breen RAM, Swaden L, Ballinger J, Lipman MCI. Tuberculosis and HIV co-infection - A practical therapeutic approach. Drugs 2006;66(18):2299-2308.
(27) Maartens G, Decloedt E, Cohen K. Effectiveness and safety of antiretrovirals with rifampicin: crucial issues for high-burden countries. Antivir Ther 2009;14(8):1039-1043.
(28) Breen RA, Miller RF, Gorsuch T, Smith CJ, Ainsworth J, Ballinger J, et al. Virological response to highly active antiretroviral therapy is unaffected by antituberculosis therapy. J Infect Dis 2006 May 15;193(10):1437-1440.
(29) Burman WJ, Gallicano K, Peloquin C. Therapeutic implications of drug interactions in the treatment of human immunodeficiency virus-related tuberculosis. Clin Infect Dis 1999 Mar;28(3):419-29; quiz 430.
(30) Tappero JW, Bradford WZ, Agerton TB, Hopewell P, Reingold AL, Lockman S, et al. Serum concentrations of antimycobacterial drugs in patients with pulmonary tuberculosis in Botswana. Clinical Infectious Diseases 2005 AUG 15;41(4):461-469.
(31) McIlleron H, Willemse M, Werely CJ, Hussey GD, Schaaf HS, Smith PJ, et al. Isoniazid Plasma Concentrations in a Cohort of South African Children with Tuberculosis: Implications for International Pediatric Dosing Guidelines. Clinical Infectious Diseases 2009 JUN 1;48(11):1547-1553.
(32) Holland DP, Hamilton CD, Weintrob AC, Engemann JJ, Fortenberry ER, Peloquin CA, et al. Therapeutic Drug Monitoring of Antimycobacterial Drugs in Patients with Both Tuberculosis and Advanced Human Immunodeficiency Virus Infection. Pharmacotherapy 2009 MAY;29(5):503-510.
(33) Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther Drug Monit 2009 06;31(3):327-336.
(34) McDade TW, Williams S, Snodgrass JJ. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography 2007 Nov;44(4):899-925.
(35) Bowen C. Automatable Processes and Future Directions in Dried Blood Spot Analysis. Am Lab 2010 OCT;42(10):11-14.
(36) Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed Chromatogr 2010 01;24(1099-0801; 0269-3879; 1):49-65.
(37) Hoogtanders K, van der HJ, Christiaans M, van de PA, van HJ, Stolk L. Dried blood spot measurement of tacrolimus is promising for patient monitoring. Transplantation 2007 01/27;83(0041-1337; 2):237-238.
(38) Leichtle AB, Ceglarek U, Witzigmann H, Gabel G, Thiery J, Fiedler GM. Potential of dried blood self-sampling for cyclosporine c(2) monitoring in transplant outpatients. J Transplant 2010;2010:201918.
(39) Li JH, Burzynski JN, Lee YA, Berg D, Driver CR, Ridzon R, et al. Use of therapeutic drug monitoring for multidrug-resistant tuberculosis patients. Chest 2004 DEC;126(6):1770-1776.
(40) Johannessen A, Troseid M, Calmy A. Dried blood spots can expand access to virological monitoring of HIV treatment in resource-limited settings. J Antimicrob Chemother 2009 Dec;64(6):1126-1129.
(41) Kredo T, Van der Walt JS, Siegfried N, Cohen K. Therapeutic drug monitoring of antiretrovirals for people with HIV. Cochrane Database Syst Rev 2009 Jul 8;(3)(3):CD007268.
(42) Parker SP, Cubitt WD. The use of the dried blood spot sample in epidemiological studies. J Clin Pathol 1999 Sep;52(9):633-639.
(43) World Health Organization. Rapid advice: Treatment of tuberculosis in children. 2010; Available at: http://whqlibdoc.who.int/publications/2010/9789241500449_eng.pdf. Accessed February, 2011.
(44) Ramachandran G, Kumar AK, Swaminathan S. Pharmacokinetics of Anti-tuberculosis Drugs in Children. Indian J Pediatr 2010 Dec 17.
(45) Graham SM. Treatment of paediatric TB: revised WHO guidelines. Paediatr Respir Rev 2011 Mar;12(1):22-26.
(46) Pandya HC, Spooner N, Mulla H. Dried blood spots, pharmacokinetic studies and better medicines for children. Bioanalysis 2011 Apr;3(7):779-786.
(47) Patel P, Mulla H, Tanna S, Pandya H. Facilitating pharmacokinetic studies in children: a new use of dried blood spots. Arch Dis Child 2010 JUN;95(6):484-487.

(48) Ette EI, Williams PJ, Lane JR. Population pharmacokinetics III: design, analysis, and application of population pharmacokinetic Studies. Ann Pharmacother 2004 Dec;38(12):2136-2144.
(49) Guthrie R, Susi A. A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants. Pediatrics 1963;32(3):338-&.
(50) Wilcken B, Wiley V. Newborn screening. Pathology 2008 Feb;40(2):104-115.
(51) Padilla CD, Therrell BL. Newborn screening in the Asia Pacific region. J Inherit Metab Dis 2007 Aug;30(4):490-506.
(52) Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens 1. J Nutr 2001 05;131(0022-3166; 0022-3166; 5):1631S-1636S.
(53) Suyagh M, Collier PS, Millership JS, Iheagwaram G, Millar M, Halliday HL, et al. Metronidazole population pharmacokinetics in preterm neonates using dried blood-spot sampling. Pediatrics 2011 Feb;127(2):e367-74.
(54) Gervasini G, Benitez J, Carrillo JA. Pharmacogenetic testing and therapeutic drug monitoring are complementary tools for optimal individualization of drug therapy. Eur J Clin Pharmacol 2010 Aug;66(8):755-774.
(55) Boer T, Wieling J, Meulman E, Reuvers M, Renkema G, Daas I, et al. Application of dried blood spot sampling combined with LC-MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. Biomed Chromatogr 2011 Feb 1.
(56) US Department of Health and Human Services Food and Drug Administration. Guidance for Industry. Bioanalytical Method Validation. ; 2001.
(57) Vu DH, Koster RA, Alffenaar JWC, Brouwers JRBJ, Uges DRA. Determination of moxifloxacin in dried blood spots using LC–MS/MS and the impact of the hematocrit and blood volume. Journal of Chromatography B 2011 5/1;879(15-16):1063-1070.
(58) ter HR, Rosing H, van Gorp EC, Mulder JW, van der Steeg WA, Beijnen JH, et al. Quantification of protease inhibitors and non-nucleoside reverse transcriptase inhibitors in dried blood spots by liquid chromatography-triple quadrupole mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2008 05/15;867(1570-0232; 2):205-212.
(59) Denniff P, Spooner N. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. Bioanalysis 2010 08/01; 2010/12;2(8):1385-1395.
(60) van der Heijden J, de Beer Y, Hoogtanders K, Christiaans M, de Jong GJ, Neef C, et al. Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography-mass spectrometry. J Pharm Biomed Anal 2009 11/01;50(4):664-670.
(61) Ntale M, Ogwal-Okeng JW, Mahindi M, Gustafsson LL, Beck O. A field-adapted sampling and HPLC quantification method for lumefantrine and its desbutyl metabolite in whole blood spotted on filter paper. J Chromatogr B Analyt Technol Biomed Life Sci 2008 12/15;876(1570-0232; 2):261-265.
(62) Ntale M, Mahindi M, Ogwal-Okeng JW, Gustafsson LL, Beck O. A field-adapted HPLC method for determination of amodiaquine and its metabolite in whole blood dried on filter paper. J Chromatogr B Analyt Technol Biomed Life Sci 2007 11/01;859(1570-0232; 1):137-140.
(63) Green MD, Mount DL, Nettey H. High-performance liquid chromatographic assay for the simultaneous determination of sulfadoxine and pyrimethamine from whole blood dried onto filter paper. J Chromatogr B Analyt Technol Biomed Life Sci 2002 02/05;767(1570-0232; 1):159-162.
(64) Koal T, Burhenne H, Romling R, Svoboda M, Resch K, Kaever V. Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2005;19(0951-4198; 21):2995-3001.
(65) Suyagh MF, Iheagwaram G, Kole PL, Millership J, Collier P, Halliday H, et al. Development and validation of a dried blood spot-HPLC assay for the determination of metronidazole in neonatal whole blood samples. Anal Bioanal Chem 2010 05;397(1618-2650; 2):687-693.

(66) Liang X, Li Y, Barfield M, Ji QC. Study of dried blood spots technique for the determination of dextromethorphan and its metabolite dextrorphan in human whole blood by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2009 03/15;877(1873-376; 8-9):799-806.

(67) Allanson AL, Cotton MM, Tettey JN, Boyter AC. Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: a potential method for therapeutic drug monitoring. J Pharm Biomed Anal 2007 08/15;44(0731-7085; 4):963-969.

(68) Deglon J, Thomas A, Daali Y, Lauer E, Samer C, Desmeules J, et al. Automated system for on-line desorption of dried blood spots applied to LC/MS/MS pharmacokinetic study of flurbiprofen and its metabolite. J Pharm Biomed Anal 2011 JAN 25;54(2):359-367.

(69) Deglon J, Thomas A, Cataldo A, Mangin P, Staub C. On-line desorption of dried blood spot: A novel approach for the direct LC/MS analysis of micro-whole blood samples. J Pharm Biomed Anal 2009 05/01;49(0731-7085; 4):1034-1039.

(70) Abu-Rabie P, Spooner N. Direct quantitative bioanalysis of drugs in dried blood spot samples using a thin-layer chromatography mass spectrometer interface. Anal Chem 2009 Dec 15;81(24):10275-10284.

(71) Majors RE. New Directions in Whole Blood Analysis: Dried Blood Spot Analysis and Beyond. Lc Gc N Am 2011 JAN;29(1):14-+.

(72) Wilhelm AJ, den Burger JC, Vos RM, Chahbouni A, Sinjewel A. Analysis of cyclosporin A in dried blood spots using liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2009 03/24(1873-376).

(73) Wilhelm AJ, den Burger JCG, Chahbouni A, Vos RM, Sinjewel A. Analysis of mycophenolic acid in dried blood spots using reversed phase high performance liquid chromatography. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2009 NOV 15;877(30):3916-3919.

(74) Mitnick C, Bayona J, Palacios E, Shin S, Furin J, Alcantara F, et al. Community-based therapy for multidrug-resistant tuberculosis in Lima, Peru. N Engl J Med 2003 01/09;348(1533-4406; 2):119-128.

(75) Antony SJ, Harrell V, Christie JD, Adams HG, Rumley RL. Clinical differences between pulmonary and extrapulmonary tuberculosis: a 5-year retrospective study. J Natl Med Assoc 1995 03;87(0027-9684; 0027-9684; 3):187-192.

(76) Fujimoto T, Tsuda Y, Tawa R, Hirose S. Fluorescence polarization immunoassay of gentamicin or netilmicin in blood spotted on filter paper. Clin Chem 1989 05;35(0009-9147; 5):867-869.

(77) Kolawole JA, Mustapha A. Improved RP-HPLC determination of quinine in plasma and whole blood stored on filter paper. Biopharm Drug Dispos 2000 12;21(0142-2782; 9):345-352.

(78) Holub M, Tuschl K, Ratschmann R, Strnadovß KA, Mnhl A, Heinze G, et al. Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. Clinica Chimica Acta 2006 11;373(1-2):27-31.

(79) Adam BW, Alexander JR, Smith SJ, Chace DH, Loeber JG, Elvers LH, et al. Recoveries of phenylalanine from two sets of dried-blood-spot reference materials: prediction from hematocrit, spot volume, and paper matrix. Clin Chem 2000 01;46(0009-9147; 0009-9147; 1):126-128.

(80) Damen CW, Rosing H, Schellens JH, Beijnen JH. Application of dried blood spots combined with high-performance liquid chromatography coupled with electrospray ionisation tandem mass spectrometry for simultaneous quantification of vincristine and actinomycin-D. Anal Bioanal Chem 2009 04/24(1618-2650).

(81) Malm M, Lindegardh N, Bergqvist Y. Automated solid-phase extraction method for the determination of piperaquine in capillary blood applied onto sampling paper by liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2004 09/25;809(1570-0232; 1):43-49.

(82) Lindegarth N, Funding L, Bergqvist Y. Automated solid-phase extraction method for the determination of atovaquone in capillary blood applied onto sampling paper by rapid high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 2001 07/15;758(1387-2273; 2):137-144.

(83) Blessborn D, Romsing S, Annerberg A, Sundquist D, Bjorkman A, Lindegardh N, et al. Development and validation of an automated solid-phase extraction and liquid chromatographic method for determination of lumefantrine in capillary blood on sampling paper. J Pharm Biomed Anal 2007 10/18;45(0731-7085; 2):282-287.
(84) Garcia BR, Henseler J, Mattern R, Skopp G. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. Ther Drug Monit 2008 12;30(1536-3694; 6):733-739.
(85) Ng K, Zhou H, Zhang YL, Hybertson B, Randolph T, Christians U. Quantification of isoniazid and acetylisoniazid in rat plasma and alveolar macrophages by liquid chromatography-tandem mass spectrometry with on-line extraction. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2007 MAR 1;847(2):188-198.
(86) Gitau EN, Muchohi SN, Ogutu BR, Githiga IM, Kokwaro GO. Selective and sensitive liquid chromatographic assay of amodiaquine and desethylamodiaquine in whole blood spotted on filter paper. J Chromatogr B Analyt Technol Biomed Life Sci 2004 01/05;799(1570-0232; 1):173-177.
(87) Li PK, Lee JT, Conboy KA, Ellis EF. Fluorescence polarization immunoassay for theophylline modified for use with dried blood spots on filter paper. Clin Chem 1986 03;32(0009-9147; 3):552-555.
(88) de Velde F, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2009 Jun 15;877(18-19):1771-1777.
(89) Cheung CY, van der HJ, Hoogtanders K, Christiaans M, Liu YL, Chan YH, et al. Dried blood spot measurement: application in tacrolimus monitoring using limited sampling strategy and abbreviated AUC estimation. Transpl Int 2008 02;21(0934-0874; 2):140-145.
(90) Hoogtanders K, van der Heijden J, Christiaans M, Edelbroek P, van Hooff JP, Stolk LM. Therapeutic drug monitoring of tacrolimus with the dried blood spot method. J Pharm Biomed Anal 2007 07/27;44(3):658-664.
(91) Bergqvist Y, al KJ, Krysen B, Berggren P,I, Rombo L. High-performance liquid chromatographic method for the simultaneous determination of mefloquine and its carboxylic metabolite in 100-microliters capillary blood samples dried on paper. J Chromatogr 1993 06/02;615(0021-9673; 2):297-302.
(92) Lejeune D, Souletie I, Houze S, Le bricon T, Le bras J, Gourmel B, et al. Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography. J Pharm Biomed Anal 2007 02/19;43(3):1106-1115.
(93) Rowland M, Emmons GT. Use of dried blood spots in drug development: pharmacokinetic considerations 1. AAPS J 2010 09;12(1550-7416; 1550-7416; 3):290-293.
(94) Jones AW, Larsson H. Distribution of diazepam and nordiazepam between plasma and whole blood and the influence of hematocrit. Ther Drug Monit 2004 Aug;26(4):380-385.
(95) Hinderling PH. Red blood cells: A neglected compartment in pharmacokinetics and pharmacodynamics. Pharmacol Rev 1997 SEP;49(3):279-295.
(96) Fleuren HL, van Rossum JM. Nonlinear relationship between plasma and red blood cell pharmacokinetics of chlorthalidone in man. J Pharmacokinet Biopharm 1977 Aug;5(4):359-375.
(97) Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. Bioanalysis 2010 Nov;2(11):1791-1796.
(98) Spooner N, Ramakrishnan Y, Barfield M, Dewit O, Miller S. Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations. Bioanalysis 2010 08/01; 2010/12;2(8):1515-1522.
(99) Mohammed BS, Cameron GA, Cameron L, Hawksworth GH, Helms PJ, McLay JS. Can finger-prick sampling replace venous sampling to determine the pharmacokinetic profile of oral paracetamol? Br J Clin Pharmacol 2010 JUL;70(1):52-56.
(100) Saracino MA, Lazzara G, Prugnoli B, Raggi MA. Rapid assays of clozapine and its metabolites in dried blood spots by liquid chromatography and microextraction by packed sorbent procedure. J Chromatogr A 2011 Feb 4.

(101) Van Schooneveld T, Swindells S, Nelson SR, Robbins BL, Moore R, Fletcher CV. Clinical Evaluation of a Dried Blood Spot Assay for Atazanavir. Antimicrob Agents Chemother 2010 OCT;54(10):4124-4128.

(102) Newman MS, Brandon TR, Groves MN, Gregory WL, Kapur S, Zava DT. A liquid chromatography/tandem mass spectrometry method for determination of 25-hydroxy vitamin d(2) and 25-hydroxy vitamin d(3) in dried blood spots: a potential adjunct to diabetes and cardiometabolic risk screening 1. J Diabetes Sci Technol 2009 01;3(1932-2968; 1932-2968; 1):156-162.

(103) Cornbleet PJ, Gochman N. Incorrect Least-Squares Regression Coefficients in Method-Comparison Analysis. Clin Chem 1979;25(3):432-438.

(104) Allanson AL, Boyter AC, Tettey JNA, Cotton MM. Determination of anti-tuberculosis drugs in blood spots. J Pharm Pharmacol 2007;59:38.

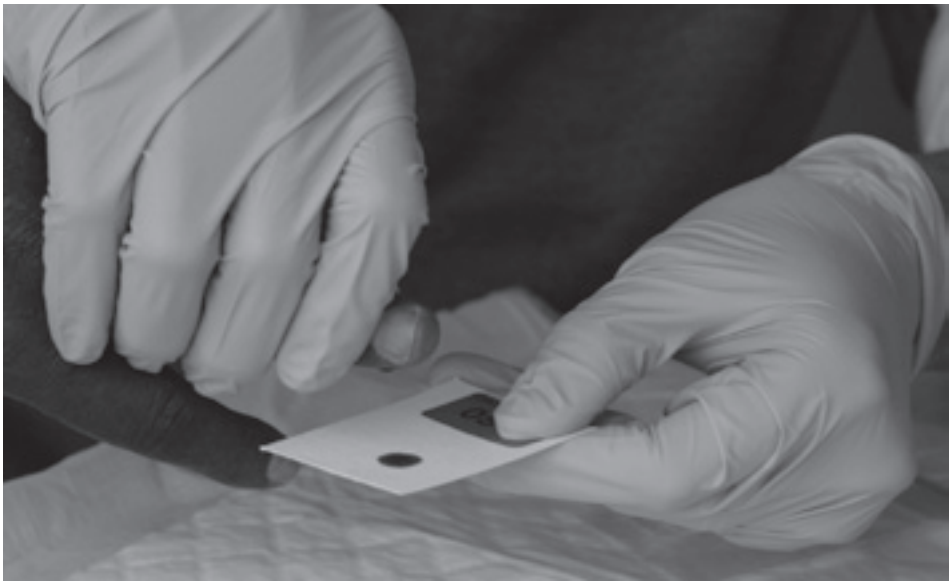


Figure 1. Dried blood spot sampling from a tuberculosis patient (following method 2)

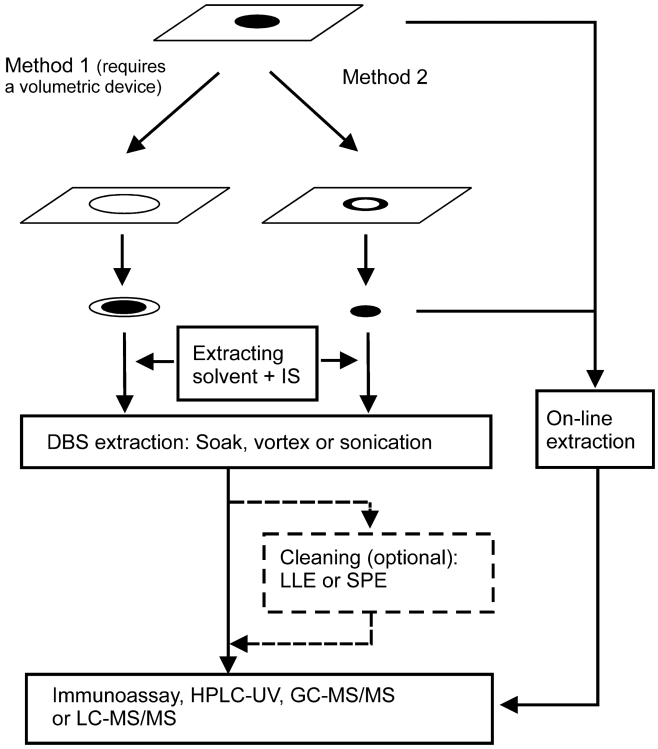


Figure 2. Dried blood spot extraction and analysis

IS: internal standard

HPLC-UV: high-performance liquid chromatography – ultraviolet detector

GC: gas chromatography

MS/MS: tandem mass spectrometry

Chapter

5

Determination of Moxifloxacin in Dried Blood Spots Using LC–MS/MS and the Impact of the Hematocrit and Blood Volume

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Determination of Moxifloxacin in Dried Blood Spots using LC-MS/MS and the Impact of the Hematocrit and Blood Volume

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ABSTRACT

Moxifloxacin (MFX) is a potential oral agent use in the treatment of multidrug-resistance tuberculosis (MDR-TB). Due to variability in pharmacokinetics and *in vitro* susceptibility of causative bacteria, therapeutic drug monitoring (TDM) of MFX is recommended. Conventional plasma sampling for TDM is facing logistical challenges, especial in limited resource areas, and dried blood spots (DBS) sampling may offer a chance to overcome this problem. The objective of this study was to develop a LC-MS/MS method for determination of MFX in dried blood spots (DBS) that is applicable for TDM.

The influence of paper type, the hematocrit (Hct) and the blood volume per spot (V_b) on the estimated blood volume in a disc (V_{est}) was investigated. The extracts of 8mm diameter discs punched out from DBS were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) with cyanoimipramin as internal standard. The method was validated with respect to selectivity, linearity, accuracy, precision, sensitivity, recovery and stability. The effect of Hct and V_b on LC-MS/MS analytical result was also investigated. The relationship between MFX concentrations in venous and finger prick DBS and those in plasma was clinically explored.

V_{est} was highly influenced by Hct while the effect of V_b appeared to be different among paper types. Calibration curves were linear in the range of 0.05-6.00 mg/L with inter-day and intra-day precisions and biases of less than 11.1%. The recovery was 84.5, 85.1 and 92.6 % in response to blood concentration of 0.15, 2.50 and 5.00 mg/L, respectively. A matrix effect of less than 11.9% was observed. MFX in DBS was stable for at least 4 weeks at room condition (temperature of 25°C and 50% of humidity). A large range of Hct value produced a significant analytical bias and it can be corrected with resulting DBS size. A good correlation between DBS and plasma concentrations was observed and comparable results between venous DBS and finger prick DBS was attained. This fully validated method is suitable for determination of MFX in dried blood spot and applicable for TDM.

INTRODUCTION

Fluoroquinolones play a crucial role in MDR-TB treatment regiment (1). Moxifloxacine (MFX) is one of the most promising drug of this group with high *in vitro* and *in vivo* activity and is well tolerated (2-4). Furthermore, MFX may be useful in extensively drug-resistant tuberculosis (XDR-TB) (5). MFX appeared to be effective in shortening tuberculosis treatment if it is added to or substituted for an agent in the standard regimen (6). In selected patient populations (e.g. HIV and tuberculosis meningitis), MFX is a potential candidate to become part of the routine treatment (7,8).

Although MFX is frequently used in the treatment of tuberculosis, the optimal dosage of MFX in tuberculosis treatment is not clearly defined (9). The *in vitro* pharmacodynamic infection model showed optimized efficacy using a dosage of 800 mg per day, while 400 mg per day is given in daily practice (1,7,9). Furthermore, a significant drop in area under the curve (AUC) of MFX of approximately 30% is observed if rifampicin as an enzyme inducer is administered concomitantly (10). Taking these facts in consideration, a TDM may help to avoid too low blood levels and to improve the treatment outcomes (11). Unfortunately, facilities for determination drug level in remote rural areas are not available. As blood samples are unstable at room temperature and cooled shipment is not feasible, the application of DBS sampling is a potential solution to overcome these logistical problems. Although MFX is stable in plasma at the room temperature for at least 5 days (12) long distance transport may take more time or room temperature may be exceeded during transport. DBS sampling has also other advantages including easily to perform; lower risk of infection and the required blood sample volume is smaller (13,14). Although the influence of Hct and volume of the bloodspot (V_b) were emphasized as potential confounding factors, these may vary for each drug. Therefore Hct and Vb need to be investigated in DBS method development (13-19).

Up to now, DBS method had been developed for the pharmacokinetics and TDM of a number of drugs (13,14,20). As DBS proved its value in TDM of HIV drugs it may also help to optimize the treatment of tuberculosis (TB), especially with MDR-TB.

Clinical validation is highly recommended for DBS method development because Hct, viscosity, and consistence of blood may vary between patients. In the clinical validation the relation between the plasma concentration and the concentration of the drug in whole blood (plasma-blood cell partition coefficient) can be determined. The result obtained with DBS can be translate to the reference value which have been determined in serum or plasma value using the blood/plasma ratio (13).

The objectives of our study are to develop a LC-MS/MS method for determination of MFX in DBS, and to investigate the effect of influencing factors of this method of sampling and matrix on DBS method development.

MATERIALS AND METHODS

Chemicals and reagents

Moxifloxacin hydrochloride was provided by Bayer AG (Berlin, Germany). The internal standard, cyanoimipramine, was supplied by Roche (Woerden, The Netherlands). Acetonitrile (ACN) Lichrosolve and water for LC/MS were purchased from BioSolve (Valkenswaard, The Netherlands). The chemicals, including methanol (MeOH) Lichrosolve and trifluoroacetic anhydride, were of HPLC or analytical

grade and were obtained from VWR (Amsterdam, The Netherlands). Three types of paper, including Whatman 31 ET CHR, Whatman 903 and Whatman N_o3 were used. The punching machine (punch diameter - 8mm) was supplied by Technical Support Facilities of the University of Leiden (NL) by P.M. Edelbroek PhD, (Heemstede, the Netherlands) (13). Packed cells and pooled human serum were provided by the Department of Haematology, University Medical Center Groningen according to local regulations.

Sample preparation

Stock solutions of MFX were prepared as stock A and stock B by dissolving MFX HCl in water at concentration of 200 mg/L in order to make calibration standards and quality control (QC) samples. The stock solutions were diluted to working stock solutions of 10 mg/L (working stock A1 and B1).

Packed red blood cells (RBC) were centrifuged and the preserving solution was discarded. The subsequent cells were washed three times with physiological buffer and one time with serum before adding a precise volume of serum to produce pooled blood with the Hct of 20, 35 and 50%.

Calibration blood was prepared at concentrations of 0.05, 0.15, 0.50, 1.00, 2.00, 3.50, 5.00 and 6.00 mg/L by adding stock solution A or A1. QC samples, including LLOQ (lower limit of quantitation), LOW, MED, HIGH, OC (over the calibration curve) at concentration of 0.05, 0.15, 2.50, 5.00 and 10.00 mg/L, respectively, were prepared from stock solution B and B1 by the same method. All tests for the validation were performed using the Hct of 35% except for the evaluation of the Hct effect itself.

To prepare a DBS, 50 μL of blood was transferred onto paper by an Eppendorf pipette. It was left to dry for at least 3 hours at room temperature and then preserved in a sealed plastic bags at -80°C. Each QC level was prepared in six folds of which five were analyzed.

The extracting solution consisted of cyanoimipramine 0.03 mg/L in a mixture of methanol and water (9:1, v/v). An 8 mm diameter disc was punched out from the DBS and was then transferred to an 1.5 mL Eppendorf tube where 300 μL of extracting solution was added. The extraction was accelerated by 60 minutes of sonication. After sonication 200 μL of the extract was transferred to a polypropylene vial and 5 μL was injected into the LC-MS/MS system.

Equipment and conditions

All samples were analyzed on a Thermo Fisher Scientific TSQ Quantum Discovery (Waltham, US) triple quadrupole LC-MS/MS with a Thermo Surveyor MS pump and a surveyor plus autosampler with a set temperature of +20°C. Analyses were performed on a 50 mm x 2.1 mm HyPurity C_{18} 5-μm analytical column (Interscience Breda, the Netherlands). The mobile phase at flow rate of 0.3 mL/min consisted of purified water, acetonitrile and an aqueous buffer (containing ammonium acetate 10 g/L, acetic acid 35 mg/L and trifluoracetic anhydride 2 mL/L water). The buffer was maintained constant at 5% during the gradient (Table 1).

The Thermo TSQ Quantum Discovery mass selective detector was used in positive ion mode and performed selected reaction monitoring as scanning mode. The mass parameters of m/z 402.0 - 358.2 (collision energy 19 eV) and *m/z* 306.0 - 218.0 (collision energy 39 eV) were measured with scan width of 0.5 m/z for MFX and cyanoimipramine detection. Ion spray voltage, sheath gas pressure, auxiliary gas pressure and capillary temperature were set at 3500 V, 35 arb (arbitrary units), 5 arb and 350°C,

respectively. Peak height integration and quantification of the components were achieved with Xcalibur software version 1.4. SRI (Thermo Fisher Scientific, Waltham, US).

METHOD DEVELOPMENT AND VALIDATION

Method development

To determine MFX in a DBS, a fixed diameter disc is punched out from DBS. Normally, it is assumed that blood volumes in different discs are equal. However, paper type, blood viscosity, that mostly relates to Hct, and V_b, were possible factors introducing analytical bias (13,14,19). As a consequence, we evaluated the variation in disc-weight and blood spreading with three types of paper including Whatman grade 3, Whatman 31ET CHR and Whatman 903.

From each of six paper cards taken randomly, six blank paper discs were punched out and scaled to determine disc-weight variation. In addition, a precise blood volume of 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 μL with a Hct of 20, 35 and 50% was pipetted on the paper in 6 fold. From both sides of the blood spots, images were taken using a Nikkon D60 camera. Areas of blood spots were measured by image analysis using ImageJ® software (version 1.42q). The V_{est} was calculated using the equation:

$$V_{est} = (1/4).\pi.d^2.V_b/S_{DBS} \quad (1)$$

In Eq. (1), *d* is diameter of the punch (8 mm); V_b and S_{DBS} are blood volume and respective DBS area. For each type of paper, linear regression analysis was performed to estimate the effect of the two parameters, Hct and V_b, on the variation of V_{est}. The changes of V_{est} over the investigated range of Hct and V_b were calculated as $Error_{Hct} = \Delta_{Hct} b_{Hct}/\overline{V}_{est}$ and $Error_{Vb} = \Delta_{Vb} b_{Vb}/\overline{V}_{est}$, respectively. In which, $\overline{V}_{est}$ is the average of V_{est}. Δ_{Hct} and Δ_{Vb} are the differences between the lowest and the highest values of Hct and Vb that were investigated, respectively; b_{Hct} and b_{Vb} are unstandardized regression coefficients of Hct and V_b. To develop the extraction method, different extracting solutions

including MeOH, ACN, water, the mixture of ACN:MeOH (16:84, v/v) and the mixtures of MeOH:water (10:90 and 20:80, v/v) were tested with a sonication time of 30min. The extracting solution was selected based on the visual signs and the chromatographic response of the extraction. With this extracting solution, the sonication time of 0, 10, 30, 60 and 100 min was evaluated and the optimal value was selected. The DBS at concentration of 2.5 mg/L was used in the method development.

Method validation

The method was validated in terms of linearity, selectivity and specificity, accuracy, precision, dilution integrity, carry-over, process efficiency and stability (21). Validation was performed with a maximum tolerated bias and coefficient of variation (CV) of 20% for the LLOQ and 15% for the other validation concentrations. In addition, the influence of Hct and Vb were also evaluated.

Oneach of three consecutive days, a single calibration curve with eight concentration levels was analyzed. Calibration curves were then established using 1/x weighted linear regression. Peak height ratios of MFX and the internal standard were used to calculate concentrations. Inter and intra-day reproducibility was evaluated at LLOQ, LOW, MED, HIGH and OC levels. The extract at OC level was diluted ten times with the extract of a blank DBS and the analytical result was then multiplied by ten to correct for the dilution. Selectivity and specificity were evaluated by analyzing blank and LLOQ

DBS samples prepared from five different batches of real human blood. The carry-over was estimated by injecting a blank sample five times after analyzing a HIGH-level sample. To calculate the process efficiency, 10_L of blank and QC (LOW, MEDand HIGH) bloodwereused tomakea spot. The whole spotwas punched out and extracted. Process efficiency, which was defined as matrix effect and recovery, was calculated from peak height responses of three solutions (A, B and C). Solution Awas the extract of 10_L-QC DBS in extracting solution. Solution B was the mixture of MFX stock solution and the extracting solution at concentrations equal to nominal values of solution A. Solution C was the extract of 10_L-blank DBS which was extracted by solution B. The matrix effect and recovery were determined as: matrix effect = (C–B)/B; recovery = A/C (14).

In addition, the matrix effect was also investigated by injecting the extracts of five DBS samples derived from finger pricks of MFX free volunteers while MFX and cyanoimipramine neat solution were post-column infused (22).

The stability of processed samples after 24 and 48 hours stored in the auto-sampler was evaluated by re-injecting the extracts of previous days and calibrated by a freshly prepared calibration curve. Long-term stability was investigated for - 80°C, room condition (25°C and 50% of humidity), high temperature (50°C) and high humidity (~100% at room temperature) at 2 weeks and 4 weeks after DBS preparation. High humidity environment was created by storing DBS in a sealed plastic bag with wet tissues without contacting with DBS paper and monitored by a hygrometer.

Influence of Hct and V_b

Tuberculosis patients have a relatively low Hct value of approximately 35 ± 6 % (23). For this reason, Hct values of 20, 25, 30, 35, 40, 45 and 50 % were evaluated using the same experiment described in Section 2.4.1. A linear regression equation between V_{est} and Hct was constructed. Furthermore, the QC level of LOW, MED and HIGH at each Hct value were prepared, analyzed and calibrated by calibration samples with a Hct of 35 %. The result was then corrected by the following equation:

$$C_{corrected} = C_{observed} \quad \frac{V_{std}}{V_{std} + b \times (Hct - 35)} \quad (2)$$

$C_{corrected}$ is the concentration after correcting for Hct; $C_{observed}$ is the concentration before correcting for Hct; V_{std} is theV_{est} at standardized Hct (35%); Hct is the Hct of corrected sample; *b* is the regression coefficient between V_{est} (mL) and Hct(%)

Blood volumes of 30, 50 and 100 μL were used to make different DBS sizes. Discs were punched out from the central part of the DBS and analyzed in five fold. Calibration samples with a blood volume of 50 μL were used to calculate the influence of V_b.

Clinical validation

The samples for clinical validation were taken from tuberculosis patients who received MFX 400 mg once daily orally as part of their treatment .The study protocol was approved by the local institutional ethics committee. Written informed consent was obtained from the patients. Venous blood sampling with a volume of about 3 ml was performed by nurses before the intake of the drug and at 1, 2, 3, 4 and 8 h after oral administration. At each sampling time, a venous DBS was prepared by pipetting 50 μL of the venous blood onto the paper. The remaining venous blood was centrifuged at 3000 rpm and the plasma was withdrawn and stored at - 20°C until analysis. In addition, at the time of pre-dosing, 2 and 8

h post-dose, finger pricks were taken and blood was dropped directly on the paper to make the finger prick DBS. The DBS were left dried for at least 3 h then stored in a sealed plastic bag at -80°C before analysis. The DBS samples were analyzed using the developed method and the plasma samples were analyzed by the routine analytical method in our laboratory (12). The correlations between venous DBS, DBS from finger prick and plasma concentrations were evaluated by simple linear regression and Passing-Bablok regression with the help of Analyse-it® software.

RESULTS

Method development

The evaluation of the different types of filter paper showed that disc weight relates to the paper's thickness and its matrix. The variation in disc weight may contribute to the variation of V_{est} and subsequently to the analytical result. Among the three types of paper tested the Whatman 31ET CHR showed the smallest variation in disc weight with a CV of 2.3% (Table 2).

With the assumption that blood equally spreads from the centre to peripheral of the DBS, V_{est} apparently represents blood volume in a punched disc. Linear regression analysis showed that Hct and V_b highly contributed to the variation of V_{est} but was not equal among different types of paper. Whatman 31ET CHR showed the highest R^2 of 0.89 which means 89% of variation in V_{est} can be explained by the regression model for this type of paper. Furthermore, the effect of the V_b was of no significant influence (β_{Vb} =0.03, p- 0.246) suggesting that blood equally spreads on this paper regardless the size of DBS. In contrast, Hct is an important predictor which explains 88% (equal to β^2_{Hct}) of total variation of the V_{est}. If the results are corrected for Hct, the accuracy of the DBS method can be improved. For the other two types of paper the lower R^2 value suggested that the variation in V_{est} is less explained by the Hct and V_b. In addition, the contributions of these parameters are not unique because β_{Hct} and β_{Vb} vary among different paper types (Table 2).

The difference in Hct ranged from 20 to 50%. This produced an $Error_{Hct}$ of 12.8% with Whatman N_o3, 22.5% with Whatman 31ETCHR and up to 26.8% with Whatman 903. With Whatman 31ET CHR the $Error_{Vb}$ was only 0.9% and this suggests the bias was not explained by V_b. However, with Whatman N_o3, the bias caused by V_b can reach 9.8%. The Whatman 31ET CHR showed a high correlation between the bias of V_{est} and Hct. Based upon these results the Whatman 31ET CHR was selected for the method development.

The extract in water was dark and not suitable for injecting into the LC-MS/MS system. We did not intend to develop another step to clean this extract because it required a more complicated procedure. In addition, MFX appeared to be poorly extracted by ACN or the mixture of ACN:MeOH (16:84 v/v) because a very low response was observed. The extract in MeOH was clear and produced a high chromatographic response. By adding 10 or 20% percent of water, the chromatographic response was improved. However, with 20% water, the extract became darker and therefore the mixture of 10% MeOH was selected. During the optimization of the extraction peak height responses increased with increasing sonication time. The maximum extraction performance was achieved at 60 minutes, where no significant difference was observed between 60 and 100 min (p - 0.43) (Fig. 1). Therefore, 60 min of sonication was selected to be used in the method validation.

METHOD VALIDATION

Selectivity and interference
No interfering peaks at the retention time of MFX and cyanoimipramine were observed in the chromatograms of 5 blank blood DBS samples. The responses of blank DBS samples were lower than 4% of those of the LLOQ DBS. These results showed that the method is selective and specific (Fig. 2).

Linearity, accuracy and precision
Calibration regression lines (n=3) were linear in the range of 0.05 - 6 mg/L with correlation coefficients (R^2) of 0.9986 ± 0.0015. The attained regression equation is:

$$y = 0.1376 \times (SD - 0.0156 \times x + 0.00026 \times (SD - 0.0014)$$

The results of inter and intra-day reproducibility, with respect to bias and precision were within accepted range for all QC levels, with a maximum bias of -8.1% and a maximum CV of 8.7%. The maximum bias and CV of the OC samples after correcting for the dilution were -11.1% and 5.8%, respectively (Table 3).

During method development, carry-over was observed and re-injections of blank samples for at least 4 times were needed to totally eliminate the carry-over effect. Consequently, 5 injections of blank sample were used after a HIGH level sample to resolve carry-over during validation.

Recovery
High recoveries of 84.5%, 85.1% and 92.6% for QC LOW, MED and HIGH were achieved and no significant matrix effect was observed (Table 4). Furthermore, no significant ion suppression or ion enhancement visually presented at the retention time of MFX (1.5 min.) or cyanoimipramine (2.0 min.) during ion suppression testing with post-column infusion (Fig. 3).

Stability
The stability of processed samples in the auto-sampler was evaluated at 24 and 48 hours and complied with the validation criteria. For long-term stability, the DBS samples were stable at room conditions for 2 and 4 weeks. However, high humidity and high temperature significantly accelerated the degradation of MFX in DBS as the MFX amounts decreased up to -33.5% bias (Table 5). This indicates that the samples should not be exposed to high temperatures and extremely high humidity.

Influence of Hct and V_b
The regression equation between V_{est} and Hct was:

$$V_{est} = 19.98 + 0.1398 \times (Hct - 35) \quad (R^2=0.81)$$

From this result, V_{std} of 19.98 and b of 0.1398 were applied to Eq. (2). Extreme Hct percentages showed high analytical biases before correcting. The difference of uncorrected concentrations between lowest and highest Hct was approximately 40%. After correcting for Hct, the biases were lowered and fell within the accepted range of 15% (Fig. 4).

The Vb showed to have an effect on the concentration by less than 15% bias. The Vb showed to be

directly proportional to the concentration of the DBS. Even though the volume of the DBS has a minor effect on the concentration of the punched area of the DBS, it does affect the accuracy of the analysis within validation requirements (Fig. 5).

Clinical validation

For clinical validation, the plasma, venous DBS and DBS from finger prick samples were taken from 6 tuberculosis patients with hematocrit values of 26, 37, 33, 41, 35 and 38%. The simple linear regression showed excellent correlations between the plasma level and the DBS level: finger prick DBS, R^2- 0.966 (n - 18); venous DBS, R^2 - 0.973 (n - 36). Using a Passing Bablok regression, the obtained slopes of regression lines between DBS and plasma concentrations was significantly higher than 1 (95% CI: finger prick DBS, 1.32 - 1.77; venous DBS, 1.50 - 1.66), and thus shows systemic differences between DBS and plasma concentrations (Fig. 6). A comparable result between finger prick DBS and venous DBS concentrations was observed: y - 1.01 x - 0.05 (95% CI slope: 0.92 to 1.11 and intercept: -0.23 to 0.01).

DISCUSSION

We developed a method of analysis for routine monitoring of MFX using dried blood spot sampling. Our method was based on punching and extracting a part of the collected blood spot using LC/MS/MS. Validation was performed according to the guidelines for bioanalytical method. Effect of Hct and Vb were evaluated as part of this validation. The method is suitable for clinical pharmacokinetic studies and routine monitoring of MFX in daily practice. To the best of our knowledge this is the first described validation using DBS for MFX.

The Hct value and blood volume showed to have a relation with the size of blood spot. Blood with a high Hct shows an increased viscosity resulting in a smaller bloodspot. This factor needs to be addressed if the blood is not obtained with a volumetric capillary and only part of the spot is extracted and analyzed. However, sampling with a finger prick without volumetric capillary is easier and cheaper and can therefore be preferred in case of sampling in remote rural areas. The impact of Hct and V_b on the accuracy of DBS analysis should be emphasised during method validation. Our results showed that with a Hct range from 20 to 50%, a significant difference of 22.5% in V_{est} was observed and therefore results should be corrected for Hct value. The correction can help to reduce the bias but seems not to totally eliminate it. The remaining small bias may be the result of other factors such as chromatographic effect in which the higher Hct, the higher concentration of MFX in the central part of DBS (13,14). Wilhelm et. al. concluded that Hct ranged from 20 to 70% produced no significant bias of cyclosporin A concentration in DBS (15). Nevertheless, even though all biases were less than 15%, Hct of 20 and 70% seemed to produce a higher bias. In the clinical application of DBS, Marca et. al. suggested that ignoring the impact of Hct could lead to a serious error especially if Hct levels not within the normal range (20).

In case of sampling in remote rural areas, patients' Hct values are not always available and in that case analytical bias caused by Hct seems unavoidable. However, as TB patients have a mean Hct value of 35% and our method is calibrated on this point, the resulting difference on the concentration of MFX is generally small.

In a clinical setting, it is difficult to control the size of the DBS without using a volumetric device. The effect of the size of the DBS on the bias of the result appeared to have less impact than the effect of

the Hct. The concentration of the DBS showed to be directly proportional to the blood volume it was created with. Although the volume of the bloodspot showed to be of influence, the differences were well within 15% bias (Fig. 5). Because the discs were punched out from the central part of the DBS, the possible reason could be the chromatographic effect of the paper in which MFX distributed more in the central and less at the peripheral part. This experiment proved that it is best to create bloodspots that show low variation in volume. On the other hand variation in volumes of 30-100 μL between patient blood spots will still be within validation requirements.

An ion enhancement caused by ethylene diamine tetraacetic acid (EDTA) on MFX analysis was reported earlier (12). We also experienced ion enhancement with DBS prepared from fresh frozen plasma that contained citrate as anticoagulant. For that reason, we selected serum to prepare DBS in which a matrix effect of less than 15% was observed.

In the clinical validation, it appeared that the MFX concentration in DBS was significantly higher than in plasma. This can be explained by the unequal distribution of MFX between plasma and blood cells caused by a difference in binding capacity to plasma proteins and blood cells (24). The slope of the regression lines of 1.49 (95% CI: 1.32-1.77) for finger blood/plasma ratio and 1.59 (95% CI: 1.50 to 1.66) for venous blood/plasma ratio showed a higher concentrations in blood than in plasma. Despite these differences, excellent regression correlations were observed. The ratio can be used to translate the DBS concentration into a plasma concentration. Comparable MFX concentrations between DBS from finger prick and veninpuncture were observed and thus suggested a similar MFX concentration between the venous and the finger capillary blood. This result also confirmed that the DBS sampling from the finger prick blood without a volumetric device was as reliable as using pipette. Although the free drug concentration of MFX cannot be calculated based on a DBS result, it still enables the attending physician to make clinical decisions on dosing MFX in TB patients.

CONCLUSION

A rapid and fully validated LC-MS/MS method was developed for determining MFX in DBS. Vb is of minor influence compared to Hct value on the analytical result. MFX concentrations obtained with DBS are significantly higher than the plasma concentrations because of the blood/plasma ratio but show a good correlation. As MFX is stable in DBS at room conditions for at least 4 weeks, the method can facilitate pharmacokinetic studies and TDM of this drug in remote rural areas.

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REFERENCES

(1) WHO global Tuberculsois.Stop TB Dept. Treatment of tuberculosis guidelines [WHO/HTM/TB/2009.420]. : World Health Organization; 2010.

(2) Rustomjee R, Lienhardt C, Kanyok T, Davies GR, Levin J, Mthiyane T, et al. A Phase II study of the sterilising activities of ofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. Int J Tuberc Lung Dis 2008 02;12(1027-3719; 1027-3719; 2):128-138.

(3) Yew WW, Leung CC. Management of multidrug-resistant tuberculosis: Update 2007. Respirology 2008 01;13(1440-1843; 1323-7799; 1):21-46.

(4) Angeby KA, Jureen P, Giske CG, Chryssanthou E, Sturegard E, Nordvall M, et al. Wild-type MIC distributions of four fluoroquinolones active against Mycobacterium tuberculosis in relation to current critical concentrations and available pharmacokinetic and pharmacodynamic data. J Antimicrob Chemother 2010 05;65(1460-2091; 0305-7453; 5):946-952.

(5) Aggarwal D, Mohapatra PR, Janmeja AK. Treatment of extensively drug-resistant tuberculosis. Lancet 2009 01/03;373(1474-547; 0140-6736; 9657):26-27.

(6) Conde MB, Efron A, Loredo C, De Souza GR, Graca NP, Cezar MC, et al. Moxifloxacin versus ethambutol in the initial treatment of tuberculosis: a double-blind, randomised, controlled phase II trial. Lancet 2009 04/04;373(1474-547; 0140-6736; 9670):1183-1189.

(7) Alffenaar JW, van AR, Bokkerink HJ, Luijckx GJ, van SD, Aarnoutse RE, et al. Pharmacokinetics of moxifloxacin in cerebrospinal fluid and plasma in patients with tuberculous meningitis. Clin Infect Dis 2009 10/01;49(1537-6591; 1058-4838; 7):1080-1082.

(8) Bonora S, Mondo A, Trentini L, Calcagno A, Lucchini A, Di PG. Moxifloxacin for the treatment of HIV-associated tuberculosis in patients with contraindications or intolerance to rifamycins. J Infect 2008 07;57(1532-2742; 0163-4453; 1):78-81.

(9) Gumbo T, Louie A, Deziel MR, Parsons LM, Salfinger M, Drusano GL. Selection of a moxifloxacin dose that suppresses drug resistance in Mycobacterium tuberculosis, by use of an in vitro pharmacodynamic infection model and mathematical modeling. J Infect Dis 2004 11/01;190(0022-1899; 0022-1899; 9):1642-1651.

(10) Nijland HM, Ruslami R, Suroto AJ, Burger DM, Alisjahbana B, van Crevel R, et al. Rifampicin reduces plasma concentrations of moxifloxacin in patients with tuberculosis. Clin Infect Dis 2007 10/15;45(8):1001-1007.

(11) Li J, Burzynski JN, Lee YA, Berg D, Driver CR, Ridzon R, et al. Use of therapeutic drug monitoring for multidrug-resistant tuberculosis patients. Chest 2004 12;126(0012-3692; 0012-3692; 6):1770-1776.

(12) Pranger AD, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. Determination of moxifloxacin in human plasma, plasma ultrafiltrate, and cerebrospinal fluid by a rapid and simple liquid chromatography- tandem mass spectrometry method. J Anal Toxicol 2010;34(1945-2403; 0146-4760; 3):135-141.

(13) Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther Drug Monit 2009 06;31(3):327-336.

(14) Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed Chromatogr 2010 01;24(1099-0801; 0269-3879; 1):49-65.

(15) Wilhelm AJ, den Burger JCG, Chahbouni A, Vos RM, Sinjewel A. Analysis of mycophenolic acid in dried blood spots using reversed phase high performance liquid chromatography. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2009 NOV 15;877(30):3916-3919.

(16) Holub M, Tuschl K, Ratschmann R, Strnadovß KA, Mnhl A, Heinze G, et al. Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. Clinica Chimica Acta 2006 11;373(1-2):27-31.

(17) Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens 1. J Nutr 2001 05;131(0022-3166; 0022-3166; 5):1631S-1636S.

(18) O'Broin S. Influence of hematocrit on quantitative analysis of "blood spots" on filter paper 1. Clin Chem 1993 06;39(0009-9147; 0009-9147; 6):1354-1355.
(19) Denniff P, Spooner N. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. Bioanalysis 2010 08/01; 2010/12;2(8):1385-1395.
(20) la MG, Malvagia S, Filippi L, Innocenti M, Rosati A, Falchi M, et al. Rapid assay of rufinamide in dried blood spots by a new liquid chromatography-tandem mass spectrometric method 1. J Pharm Biomed Anal 2011 01/05;54(1873-264; 0731-7085; 1):192-197.
(21) US Department of Health and Human Services Food and Drug Administration. Guidance for Industry. Bioanalytical Method Validation. ; 2001.
(22) Jessome LL, Volmer DA. Ion suppression: A major concern in mass spectrometry. Lc Gc North America 2006;24(5):498-+.
(23) Antony SJ, Harrell V, Christie JD, Adams HG, Rumley RL. Clinical differences between pulmonary and extrapulmonary tuberculosis: a 5-year retrospective study. J Natl Med Assoc 1995 03;87(0027-9684; 0027-9684; 3):187-192.
(24) Rowland M, Emmons GT. Use of dried blood spots in drug development: pharmacokinetic considerations 1. AAPS J 2010 09;12(1550-7416; 1550-7416; 3):290-293.

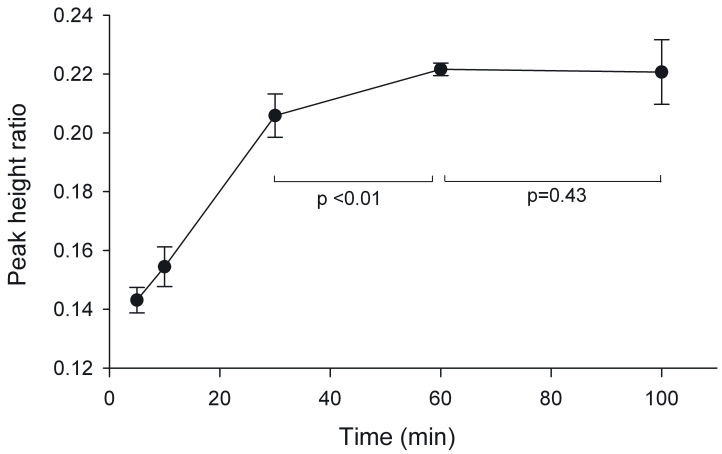

Figure 1. Influence of sonication time on the response of DBS extract (n = 5);

Result was presented as mean ± SD

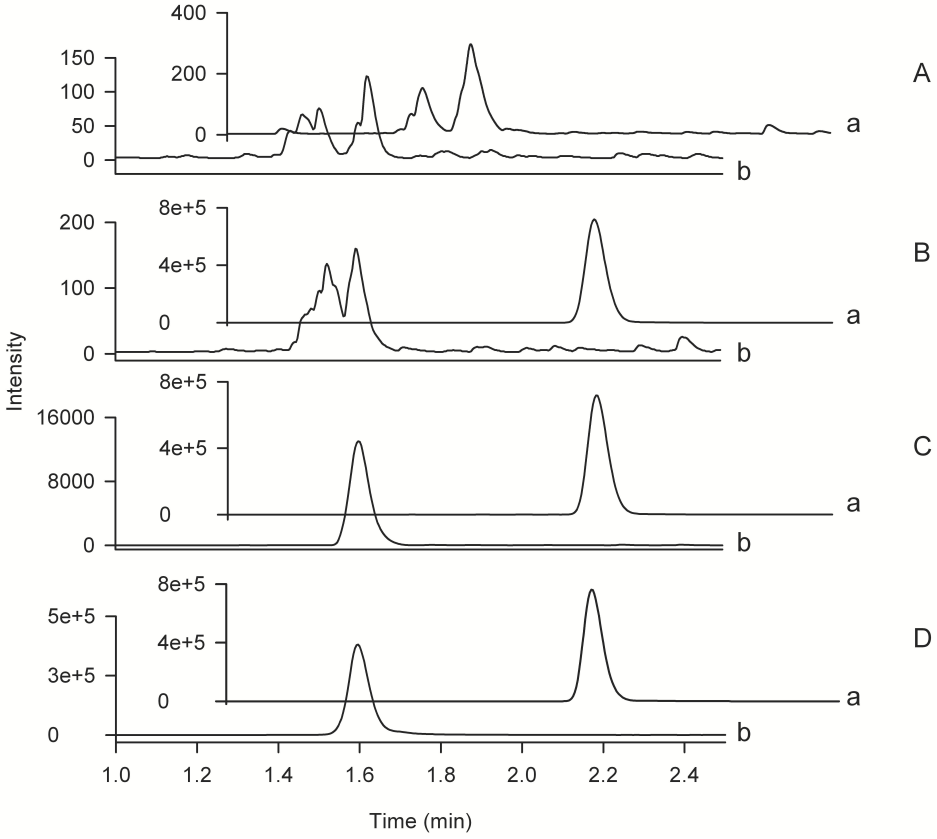

Figure 2. Represent chromatogram

Chromatogram of cyanoimipramin (a) and moxifloxacin (b) in a blank DBS extracted by blank extracting solvent (A); in a blank DBS (B), a LLOQ DBS (MFX - 0.05 mg/L) (C) and a patient finger prick DBS (MFX - 1.49 mg/L) (D) extracted by extracting solvent with internal standard (cyanoimipraimin = 0.3 mg/L).

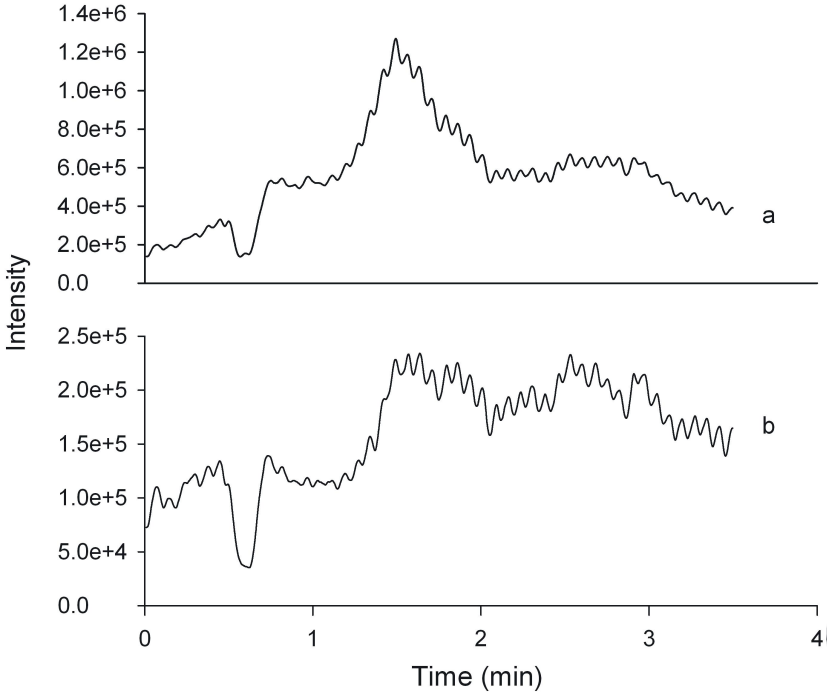

Figure 3. Representative ion suppression chromatograms obtained from post-column infusion experiments with blank DBS samples

(a) cyanoimipramin;

(b) moxifloxacin

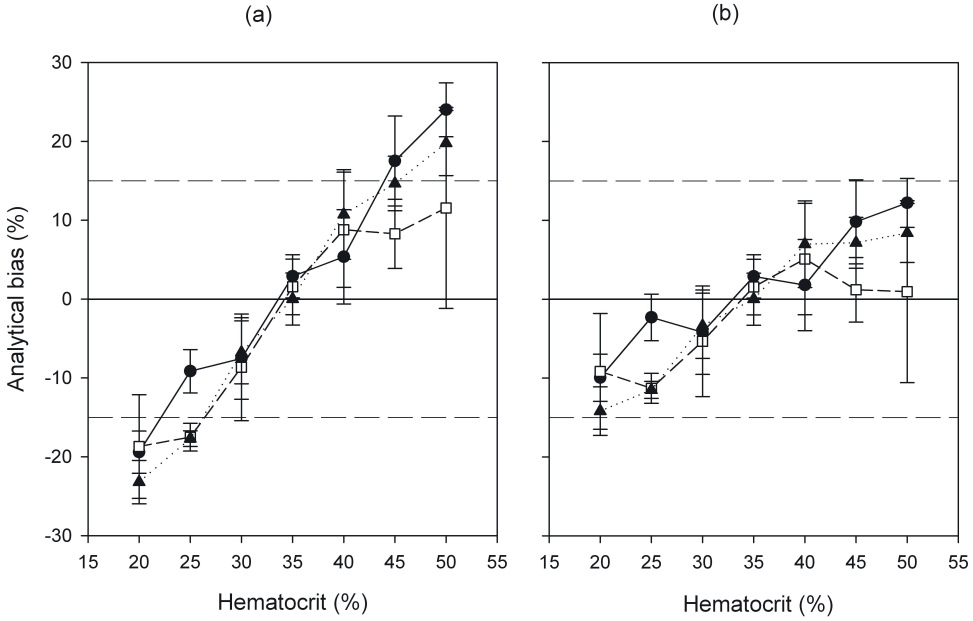

Figure 4. Influence of Hct on analytical result and the correction (n = 5)

Before correction for Hct: (a); after correction for Hct: (b)

LOW: opened square, dash line; MED: closed triangles dot line; HIGH: closed circles, solid line,

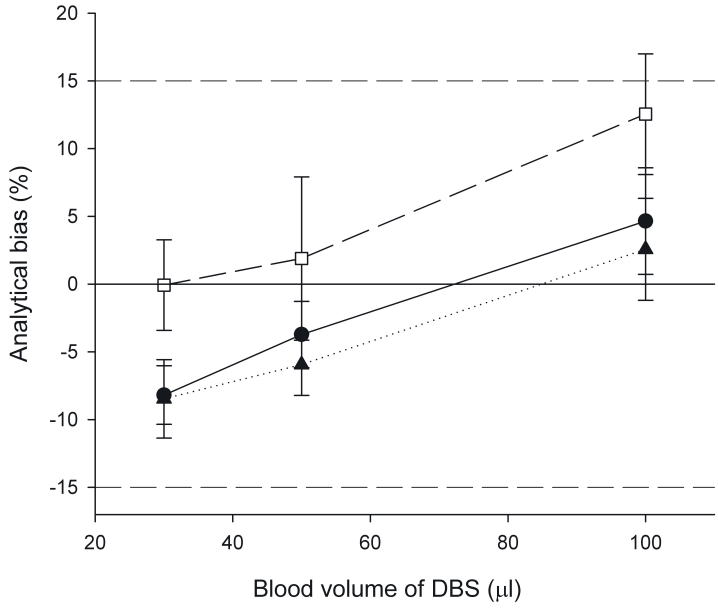

Figure 5. Influence of blood volume on analytical result (n = 5)

LOW: opened square, dash line; MED: closed triangles dot line; HIGH: closed circles, solid line

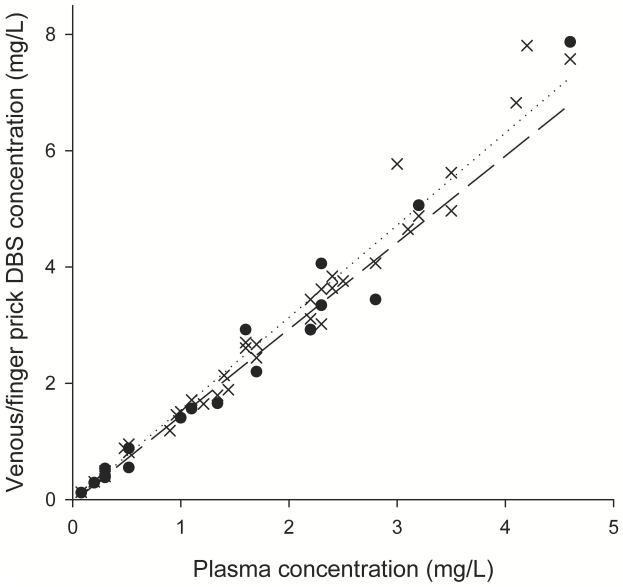

Figure 6. Correlation between MFX concentration in venous/finger prick DBS and plasma

Correlation between venous DBS and plasma concentration (cross): Passing Bablok regression line (dot line, n -36), y = 1.59x - 0.04 (95% CI slope: 1.50 - 1.66, intercept: -0.15 - 0.03); simple linear regression coefficient R2= 0.973. Correlation between finger prick DBS and plasma concentrations (closed circles): Passing Bablok regression line (dash line, n- 18), y = 1.49 x - 0.05 (95% CI slope: 1.32 - 1.77, intercept: -0.30 - 0.06; Simple linear regression coefficient R2= 0.966.

Table 1. Solvent gradient program

Time (min)	buffer (%)	Water (%)	Acetonitrile (%)
0	5	95	0
0.5	5	35	60
1.6	5	35	60
1.61	5	0	95
2.9	5	0	95
2.91	5	95	0
3.5	5	95	0

Table 2. Variability in disc weight and the effect of Hct, V_b on V_{est} among 3 types of paper

Paper	Disc weight (mg)		Effect of Hct and Vb on Vest					
	Mean	CV (%)	Mean (SD)	β_{Hct}	β_{Vb}	R^2	$Error_{Hct}$	$Error_{Vb}$
N_o 3	9.10	4.1	15.87 (1.22)	0.68 *	0.41 *	0.62	12.8%	9.8%
903	9.32	3.2	18.95 (2.48)	0.84 *	0.13 *	0.72	26.8%	5.2%
31ET CHR	9.59	2.3	19.95 (1.95)	0.94 *	0.03	0.89	22.5%	0.9%

Hct was centralized at 35% and V_b was centralized at 35 (mL)

$\beta_{Hct,}$ β_{Vb}: standardised regression coefficient of Hct and V_b

R^2: total correlation coefficients of the model.

*: p <0.001

Table 3. Accuracy and precision results (n=5)

	QC level (Nominal conc.) (mg/L)	Mean conc. (mg/L)	Precision (CV %)	Accuracy (bias %)
Day1	LLOQ (0.05)	0.053	6.8	5.8
	LOW (0.15)	0.146	8.7	-2.6
	MED (2.50)	2.572	2.4	2.9
	HIGH (5.00)	5.244	1.7	4.9
	OC (10.00)	10.343	5.5	3.4
Day2	LLOQ (0.05)	0.051	4.9	2.8
	LOW (0.15)	0.138	3.5	-8.1
	MED (2.50)	2.360	3.3	-5.6
	HIGH (5.00)	4.705	2.4	-5.9
	OC (10.00)	8.892	2.3	-11.1
Day3	LLOQ (0.05)	0.050	5.4	0.3
	LOW (0.15)	0.153	2.8	2.2
	MED (2.50)	2.388	5.5	-4.5
	HIGH (5.00)	4.968	3.2	-0.6
	OC (10.00)	8.955	5.8	-10.4
Inter-day	LLOQ (0.05)	0.051	5.8	3.0
	LOW (0.15)	0.146	6.9	-2.8
	MED (2.50)	2.440	5.4	-2.4
	HIGH (5.00)	4.972	5.1	-0.6
	OC (10.00)	9.397	8.7	-6.0

LLOQ: lower limit of quantification; MED: medium; OC: over the calibration curve

Table 4. Matrix effect and recovery of the DBS method (n=5)

QC level (nominal conc.) (mg/L)	Solution	Response		Matrix effect (%) (C/B-1)	Recovery (%) (A/C)
		Mean	CV (%)		
LOW (0.15)	A	0.020	6.0	- 3.4	84.5
	B	0.024	3.7		
	C	0.024	3.8		
MED (2.50)	A	0.335	2.5	-11.9	85.1
	B	0.446	5.1		
	C	0.393	1.8		
HIGH (5.00)	A	0.676	3.3	-5.1	92.6
	B	0.769	2.0		
	C	0.730	1.2		

A, B and C = response of solution A, B and C

Table 5. Stability after 2 and 4 weeks stored in different conditions (n=5)

Condition	QC level (nominal conc.) (mg/L)	2-Week stability			4-week stability		
		Mean conc. (mg/L)	CV (%)	Bias (%)	Mean conc. (mg/L)	CV (%)	Bias (%)
- 80°C	LOW (0.15)	0.156	3.5	4.1	0.161	4.3	7.4
	HIGH (5.00)	4,886	1.8	-2.3	5.324	4.9	6.5
Normal	LOW (0.15)	0.158	4.2	5.5	0.158	6.0	5.4
	HIGH (5.00)	4,660	2.9	-6.8	5.231	6.0	4.6
50°C	LOW (0.15)	0.124	5.8	-17.1	0.112	4.5	-25.4
	HIGH (5.00)	3,869	1.4	-22.6	3.503	6.2	-29.9
Humidity	LOW (0.15)	0.117	31.9	-21.7	0.124	17.1	-17.4
	HIGH (5.00)	3,325	39.2	-33.5	4.518	14.4	-9.6

Chapter

6a

Troubleshooting carry-over of LC-MS/MS method for rifampicin, clarithromycin and metabolites in human plasma

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Troubleshooting carry-over of LC-MS/MS method for rifampicin, clarithromycin and metabolites in human plasma

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ABSTRACT

Clarithromycin and rifampicin are used for the treatment of Mycobacteria. Pharmacokinetic drug interaction is possibly due to the influence of the two drugs on the liver enzymes. Using a Hypurity Aquastar C18 column (50 mm x 2.1 mm x 5 mm) for liquid chromatography including a polar end-capped phase for the determination of clarithromycin, rifampicin and their metabolites together in plasma using LC-MS/MS resulted in a substantial carry-over. As a consequence, the throughput of the method is not assured. Using a step by step troubleshooting procedure, such carry-over was found originating from column memory effect. With the use of another type of C18 column, the carry-over is eliminated. Due to the absence of carry-over, the analytical concentration ranges are extended and are therefore more appropriate for the analysis of patient samples. The method was re-validated for linearity, reproducibility and dilution integrity.

INTRODUCTION

The combination of rifampicin (RIF) and clarithromycin (CLR) can be used to improve the treatment outcome and prevent the resistance of *Mycobacteria*. It is well reported that RIF is a strong liver enzymes inductor while CLR is an inhibitor. Several small studies suggested that RIF may reduce the CLR plasma concentration while CLR, on the other hand, elevates the RIF plasma level (1,2). For that reason, therapeutic drug monitoring of these substances may help to assure adequate drug exposure. Furthermore, the metabolism of RIF and CLR by cytochrome P450 results in active metabolites including 25-desacetylrifampicin (Dac-RIF) and 14-hydroxyclarithromycin (14OH-CLR), respectively (3,4). The analysis of these substances is therefore recommended, but is seldom implemented in analytical methods (5,6). Osward et. al. developed a method for simultaneous determination of CLR and RIF and their metabolites but not in human plasma (6). The LC-MS/MS method published by van de Velde et. al. could simultaneously determine CLR, RIF and their metabolites in human plasma (5). In this method, the authors reported a persistent carry-over in the analysis of RIF and Dac-RIF which required five blank injections to eliminate if high standard or quality control sample was eluted. Because of the carry-over, the LLOQ for RIF and Dac-RIF was 0.2mg/L which is relatively high in comparison with low plasma trough level of these substances (1,7,8). In addition, if the concentration of RIF or Dac-RIF is higher than the upper limit of quantification (ULOQ: 5 mg/L), the sample needs to be diluted, and re-analyzed. It is noticeable that the peak concentration of RIF in plasma is normally higher than 5 mg/L (1,7,8). Therefore, the carry-over should be eliminated to increase the throughput of this analytical method in routine practice.

Contamination and carry-over are common encountered problems with LC-MS/MS analyses (9). First, the contamination may occur during the sample preparation which is normally related to the extraction procedure. Second, contamination can be generated due to the auto-sampler carry-over. Third, due to secondary interactions in the column a column memory effect may be induced. (10). Dealing with the carry-over requires the combination of systemic and logical investigation (9).

Therefore the aim of the study was to detect and eliminate the carry-over and make the method of analysis more suitable for routine analysis.

METHODS

Troubleshooting the carry-over

An effort to detect and eliminate the carry-over from the auto-sampler and column was tried. A more thorough auto-sampler (e.g. needle, needle tube and needle seat) flushing and washing procedures using different solvents (acetonitrile, isopropanol and their mixtures) were tested. If the auto-sampler flushing and washing provided no improvement, the carry-over from the column was examined by using a "duplicated" solvent gradient. The previous solvent gradient was performed in 3.5 minutes as follows: 0-2 minutes: ACN from 0 to 95%, water from 95% to 0%; 2-3 minutes: ACN 95% and water 0%; 3-3.1 minutes: decrease ACN to 0% and keep eluting with water 95% until 3.6 minutes. The aqueous buffer (ammonium acetate 10 g/L, acetic acid 35 mg/L and trifluoroacetic anhydride 2 mg/L, pH 3.5) was kept at 5% during the gradient (5). The "duplicated" solvent gradient consisted of two gradients, as described before, combined in one analysis. In this way an injection is eluted by the first gradient, while the second gradient is performed without sample injection (Figure). A high concentration sample at ULOQ level (upper limit of quantification; RIF: 30mg/L; Dac-RIF, CLR and 14OH-CLR: 10mg/L) was injected and eluted.

To estimate the carry-over effect, 3 samples including a LLOQ (lower limit of quantification RIF; Dac-RIF: 0.15mg/L; CLR, 14OH-CLR: 0.05 mg/L), a ULOQ and a blank sample were subsequently injected into the LC-MS/MS system. The carry-over was defined as the percentage of responses from the blank sample to the respective LLOQ sample.

Method validation
Two different stock solutions for the calibration and quality control samples were prepared in methanol:water (1:1v/v) containing the following concentrations: RIF: 600 mg/L; Dac-RIF, CLR, and 14-OH-CLR: 200 mg/L. Subsequently, the stock solutions were diluted ten times to produce working stock solutions. All stock solutions were stored at 4°C. Calibration samples (Table 1) and quality control samples at levels of LLOQ, LOW, MED, HIGH and over the calibration curve (OC) (Table 2) were prepared by mixing appropriate amounts of stock solutions or working stock solutions with blank human plasma (received from the Hematology department of UMCG). The added volume of stock solution was less than 5% of the total sample volume.

A plasma volume of 10 µl was transferred into a glass vial with 750 µl of protein precipitation solution, which consists of cyanoimipramine as internal standard in ACN:MeOH (21:4 v/v), The sample was vortexed for 1 minute and then stored for 30 minutes at -20°C to accelerate the protein precipitation. After 1 minute of vortexing and 5 minutes of centrifuging at 11,000 rpm, 5 µl of supernatant was injected onto the Hypurity C18 column (50 mm x 2.1 mm x 5 mm). For the detection of the analytes a Thermo Fisher triple Quadrupole detector was used. The MS/MS conditions were defined in the previously published method of de Velde et al. (5).

Each day of a three day validation, a calibration curve and a set of quality control samples were analyzed. Linear regression weighted by $1/X^2$ was used to construct the calibration curve. For determination of accuracy, precision, and dilution integrity, quality control samples were prepared and measured in 5-fold. Within-run, between-run, and overall bias and coefficient of variation (CV) were calculated using a 1-way ANOVA. Maximum tolerated bias and CV was 20% for the LLOQ and 15% for the other validation concentrations (11).

RESULTS AND DISCUSSION

Trouble shooting the carry-over of the analysis method for RIF and Dac-RIF
The carry-over may come from the auto-sampler, the switching system or the LC column (10). No improvement in terms of carry-over was attained by using different kinds of flushing and washing programs and solvents to clean the auto-sampler system. Interestingly, the chromatography of "duplicated" solvent gradient presented a significant column memory effect of RIF and Dac-RIF (Figure). Because the "duplicated" solvent gradient by-passes the auto-sampler and the switching system during the elution period, it is suggested that the persisting "carry-over" in the previously published method resulted from column memory effect.

To eliminate the column memory effect, several gradients were tested using longer eluting periods. Despite of these efforts the memory effect was persistent and the carry-over peaks maintained at about 2% and 4% of the main peaks for Dac-RIF and RIF, respectively. With such high carry-over, the analytical bias of low concentration sample could be dramatically influenced if the previous sample is at high concentration. Injecting several blank samples gradually reduced the carry-over peak yet

increased the time of analysis. Extending the elution up to 6 minutes and increasing the acetonitrile elution phase reduced the carry-over up to 0.7% and 0.2% for RIF and Dac-RIF, respectively. However, short runtimes were important to ensure a high throughput of a routine analysis. Moreover, increasing the ULOQ of the original method was preferred for RIF and Dac-RIF to minimize re-analysis of over the curve patient samples. For this purpose, carry-over should be further minimized and therefore another approach should be introduced.

The method published by de Velde et. al. used a Hypurity Aquastar C18 column (50 mm x 2.1 mm x 5 mcm) for liquid chromatography (5). The polar end-capped phase added in this column might be the explanation for the observed carry-over. RIF and Dac-Rif may interact with the stationary phase result in the column memory effect. For that reason a Hypurity C18 column without polar end-capped phase was tested. Using this column, the carry-over effect observed in an analysis of the first blank sample followed after an ULOQ was dramatically reduced to less than 0.08% for all four substances. In addition, with some adjustment of the solvent gradient program, the elution time was shortened to 3 minutes while the peak shapes remained good and the carry-over was excluded. With this finding, the linear analytical range of all four substances could be extended: RIF: 0.15 mg/L – 30 mg/L; Dac-RIF: 0.15 mg/L – 10 mg/L; CLR and 14OH-CLR: 0.05 mg/L – 10mg/L.

Method validation

The method showed good linearity for all four analyzed substances. The equation of the calibration curves and the correlation coefficients are presented in Table 1. In each assay, the deviations of the calibration samples to the linear calibration curves were less than 20% for the lowest concentration and 15% for the other concentrations. It is noticeable that the method was validated with larger analytical ranges than the method published by de Velde et. al. On the one hand, no blank injection was needed to exclude the carry-over. On the other hand, the higher ULOQ levels for RIF and Dac-RIF assure the analysis of higher concentrations without the need of diluting and re-analysis of the samples. As a consequence, the new method is more practical for the analysis of real patient plasma samples.

The reproducibility presented as bias and CV were according to the FDA guidelines (11). All the bias and CV values were less than 20% at LLOQ level and less than 15% at the other QC levels. Diluting the over curve concentration sample influenced neither the accuracy nor the precision of the validated method (Table 2).

CONCLUSIONS

With the adapted method the carry-over is eliminated and blank sample injections to reduce carry-over have become redundant. The method was re-revalidated and showed to be more practical in routine analysis.

REFERENCES

(1) Alffenaar JW, Nienhuis WA, de Velde F, Zuur AT, Wessels AM, Almeida D, et al. Pharmacokinetics of rifampin and clarithromycin in patients treated for Mycobacterium ulcerans infection. Antimicrob Agents Chemother 2010 Sep;54(9):3878-3883.

(2) WALLACE R, BROWN B, GRIFFITH D, GIRARD W, TANAKA K. Reduced Serum Levels of Clarithromycin in Patients Treated with Multidrug Regimens Including Rifampin Or Rifabutin for Mycobacterium-Avium Mycobacterium-Intracellulare Infection. J Infect Dis 1995 MAR;171(3):747-750.

(3) Rodvold K. Clinical pharmacokinetics of clarithromycin. Clin Pharmacokinet 1999 NOV;37(5):385-398.

(4) ACOCELLA G. Clinical Pharmacokinetics of Rifampicin. Clin Pharmacokinet 1978;3(2):108-127.

(5) de Velde F, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2009 Jun 15;877(18-19):1771-1777.

(6) Oswald S, Peters J, Venner M, Siegmund W. LC-MS/MS method for the simultaneous determination of clarithromycin, rifampicin and their main metabolites in horse plasma, epithelial lining fluid and bronchoalveolar cells. J Pharm Biomed Anal 2011 Apr 28;55(1):194-201.

(7) Peloquin CA, Jaresko GS, Yong CL, Keung AC, Bulpitt AE, Jelliffe RW. Population pharmacokinetic modeling of isoniazid, rifampin, and pyrazinamide. Antimicrob Agents Chemother 1997 Dec;41(12):2670-2679.

(8) Ruslami R, Nijland HM, Alisjahbana B, Parwati I, van Crevel R, Aarnoutse RE. Pharmacokinetics and tolerability of a higher rifampin dose versus the standard dose in pulmonary tuberculosis patients. Antimicrob Agents Chemother 2007 Jul;51(7):2546-2551.

(9) Weng N, Halls T. Systematic troubleshooting for LC/MS/MS - Part 2: Large-scale LC/MS/MS and automation. Biopharm-Appl Technol Biopharm Dev 2002 JAN;15(1):22-+.

(10) Hughes NC, Wong EY, Fan J, Bajaj N. Determination of carryover and contamination for mass spectrometry-based chromatographic assays. AAPS J 2007 Nov 2;9(3):E353-60.

(11) US Department of Health and Human Services Food and Drug Administration. Guidance for Industry. Bioanalytical Method Validation. ; 2001.

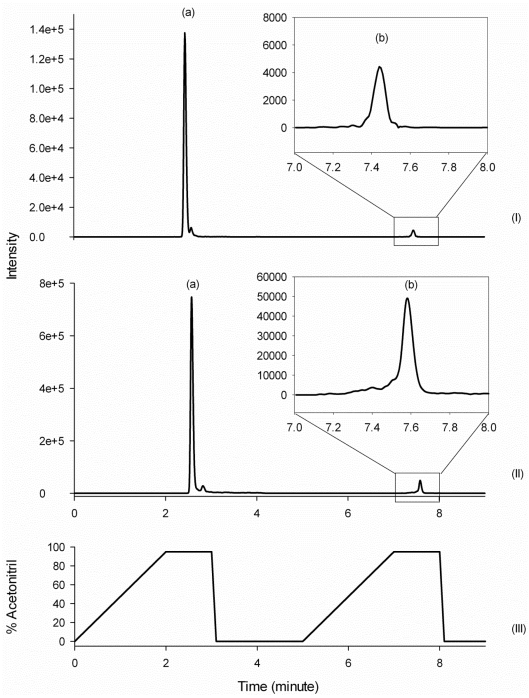

Figure.

Chromatogram of the elution of an ULOQ sample using a "duplicated" solvent gradient; (I): 25-desacetylrifampicin; (II): Rifampicin; (III): the "duplicated" gradient; (a): peaks of the first elution period; (b): Peaks due to the memory effect

Table 1. The concentrations and the inter-assay variation of the calibration curves (n=3)

Component	Calibration concentration (mg/L)	Slope ± SD	Intercept ± SD	Correlation coefficient (R)
CLR	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.421 ± 0.0065	0.0008 ± 0.0064	0.9974
14OH-CLR	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.269 ± 0.0031	-0.0020 ± 00029	0.9985
RIF	0.15, 0.45, 1.5, 3.0, 9.0, 15.0, 24.0, 30.0	0.449 ± 0.0081	-0.014 ± 0.0032	0.9971
Dac-RIF	0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.058 ± 0.0012	-0.0026 ± 0.0005	0.9957

Table 2. The accuracy, precision and the dilution integrity (n=5)

	CLR					14OH-CLR					RIF					Dac-RIF				
	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC
Nominal concentration (mg/L)	0.05	0.15	5.0	8.0	20.0	0.05	0.15	5.0	8.0	20.0	0.15	0.45	15.0	24.0	60.0	0.15	0.5	5.0	8.0	20.0
Accuracy (% bias)	-2.0	1.2	-2.0	1.6	-2.0	11.0	5.1	-0.4	6.0	-2.0	0.3	-9.6	-0.9	1.0	3.0	6.5	-9.1	2.5	10.6	4.0
Within-run precision (% CV)	7.7	3.9	2.1	4.8	5.7	7.6	6.5	2.8	2.7	3.7	5.5	4.9	3.1	3.4	5.3	9.9	6.3	1.7	3.1	4.3
Between run precision (% CV)	8.8	0.0	2.9	4.0	0.0	1.9	2.8	3.6	4.8	0.8	4.7	0.0	0.0	2.8	3.6	0.0	0.0	4.6	4.9	2.6

OC: over the calibration curve

Chapter

6b

Simultaneous Determination of Rifampicin, Clarithromycin and Their Metabolites in Dried Blood Spots Using LC-MS/MS

Submitted

Simultaneous Determination of rifampicin, clarithromycin and their metabolites in Dried Blood Spots Using LC-MS/MS

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ABSTRACT

Introduction: Rifampicin (RIF) and clarithromycin (CLR) are common drugs for the treatment of infectious like *Mycobacterium tuberculosis* and *Mycobacterium ulcerans*. The intensive and long treatment confront sub-therapeutic or toxicity because of pharmacokinetic variation, drug-drug interaction or non adherence to the treatment. Therapeutic drugs monitoring (TDM) is used to ensure efficacy and to avoid toxicity. With the application of dried blood spot (DBS), the TDM may be feasible in rural area.

Methods: An investigation focused on the interaction between RIF and the endogenous components of the DBS. A rapid analytical method was developed and validated to quantify RIF and CLR and their active metabolites desacetyl rifampicin (DAc-RIF) and 14 hydroxy clarythromcin (14OH-CLR) in DBS samples. The method was clinically validated on tuberculosis patient by comparing DBS concentration and plasma concentration

Results: The interaction between RIF with DBS matrix was competed with the present of ethylenediaminetetraacetic acid and deferoxamine, complexing agent. The competition was applied to develop a simple and fast method for simultaneous quantification of RIF, CLR and their metabolites in DBS specimen. High stability was observed as all four substances were stable at room temperature for two months. High correlation between plasma and DBS concentration RIF (r^2=0.9067) CLR (r^2=0.9785) and 14OH-CLR (r^2=0.9421) was observed.

Conclusion: The validated method is applicable for TDM of RIF and CLR and their active metabolites.

INTRODUCTION

Rifampicin (RIF) and clarithromycin (CLR) are two drugs used for the treatment *Mycobacterial* infections. According to the tuberculosis (TB) treatment guideline of World Health Organization, RIF is the back bone of the first line anti-TB drugs in the treatment of *Mycobacterium tuberculosis*. CLR is indicated for treatment of multidrug resistant (MDR) TB. In combination, RIF and CLR showed high efficacy for the treatment of *Mycobacterial ulcerans* which is the organism causing Buruli Ulcer disease (1). RIF display's large pharmacokinetic variability that may result in subtherapeutic drug exposure (2,3). It is well known that RIF is a liver enzymes inductor while CLR is an inhibitor. Several studies suggested that RIF reduced the CLR plasma concentration while CLR, on the other hand, increased the RIF plasma level (4,5). Furthermore, the metabolism of RIF and CLR by cytochrome P450 results in active metabolites including 25-desacetylrifampicin (Dac-RIF) and 14-hydroxyclarithromycin (14OH-CLR), respectively (6-8). To assure adequate drug, exposure by therapeutic drug monitoring (TDM) of these drugs may help to improve the treatment outcome.

The common endemic areas for TB or Buruli Ulcer disease often have limited resource. Conventional plasma sampling is often not feasible due to lack of equipment or cooled transportation (9). Dried blood spot (DBS) sampling has many advantages such as prolonged sample stability, lower risk of infections and transport at room temperature (9,10). These advantages may facilitate the application and implementation of TDM of drugs even in resource limited areas. Methods of analysis for the determination of RIF or CLR in the biological fluids using high performance liquid chromatography (HPLC), liquid chromatography tandem mass spectrometry (LC-MS/MS) have been reported earlier (11,12), including simultaneous determination of rifampicin and clarithromycin (14,15). Only report described the development of an analytical method to determine RIF in DBS using HPLC (13). However, the extraction method is complex showing low sensitivity and do not account for determination of DAc-RIF. The application of LC-MS/MS with high selectivity and sensitivity can help to deal with these limitations. The development of a dried blood spot analysis method is confronted with challenges that need to be solved during method development and validation. DBS samples can be extracted by hydrophilic or hydrophobic organic solvents (16-18). Aqueous extraction has a drawback that it extracted endogenous components from the DBS as well (19). The endogenous components in the blood may cause unexpected interactions or matrix effects during the analysis (21). The application of ethylenediaminetetraacetic acid (EDTA) to precipitate endogenous components has been successfully applied before (20). Another complication compared to plasma analysis is that RIF can form chelate complexes with ferric ions or can bind with hemes which are potential presented in the extract of dried blood spot (20,22). In addition to the standard validation criteria, the influence of hematocrit value and blood spot volume should be assessed during the method validation (9,10,23). Furthermore, before DBS is implemented in daily routine, the correlation between RIF DBS and plasma concentrations should be demonstrated (9,10,23). The aim of this study is to develop a rapid LC-MS/MS method for determination of RIF, CLR and their metabolites in DBS which is suitable for TDM clinical pharmacokinetic studies in rural areas.

MATERIALS AND METHODS

Chemical, reagent and disposables

Clarithromycin ($C_{38}H_{69}NO_{13}$) and 14-hydroxyclarithromycin ($C_{38}H_{69}NO_{14}$) were provided by Abbott (IL, USA). Rifampicin ($C_{43}H_{58}N_4O_{12}$) and 25-desacetylrifampicin ($C_{41}H_{56}N_4O_{11}$) were provided by Sanofi-Aventis (Frankfurt, Germany). The $^2H_8$-Rifampicin and cyanoimipramine were supplied by Brunschwig Chemie (Amsterdam, The Netherlands) and by Roche (Woerden, The Netherlands), respectively. Water and acetonitrile of ultra LC/MS grade were supplied by Biosolve (Valkenswaard, The Netherlands). Disodium ethylenediaminetetraacetic acid (EDTA), ammonium acetate, acid acetic and trifluoroacetic anhydride were of analytical grade and purchased from VWR (Amsterdam, the Netherlands). Deferoxamine mesilate was attained from Novartis Pharma (Arnhem, the Netherlands). Pooled plasma and packed red blood cells were achieved from Department of Hematology, University Medical Center Groningen according to local regulations. The Whatman 31 ET CHR paper sheet was cut in to 4 6 cm paper cards and used for the preparation of calibration and quality control (QC) DBS and patient sampling.

Sample preparation

Prepare the stock solutions

Two separate stock solutions were prepared in water at a concentration of 600 mg/L for RIF and 200mg/L for the other three analytes. These stock solutions were used to prepare the calibration curve and QC samples. For spiking low concentrations, the stock solutions were diluted with water to 25 mg/L. This ensures that the spiked volume of the stock solution could not exceed 5% of the total volume. The stock solutions were stored at 4°C.

Preparation of the Calibration Curve and QC in Blood

The packed red blood cells (RBC) achieved from the hematology department contained a preservation solution. To completely remove the preserving solution the RBC were centrifuged at 4000 rpm for 10 minutes and the upper layer was discarded. To wash the RBC, an equal volume of phosphate buffered saline with pH 7.4 was added and mixed by a rotating mixer for 5 minutes. The RBC suspension was centrifuged and the upper layer was discarded. The RBC washing procedure was subsequently repeated twice with phosphate buffered saline and lastly with plasma. The RBC was mixed with plasma and stock solution to obtain calibration and QC blood at the desired hematocrit values. All the calibration and QC blood samples were prepared at the standardized hematocrit value of 35%. The concentrations of each component in the calibration and QC samples are presented in Table 1.

Prepare the DBS Samples

The calibration and QC DBS samples were prepared by pipetting 50 µl of blood onto the paper card and left to dry for 3 hours at ambient temperature. DBS cards were stored separately in a sealed plastic bag with a desiccant sachet at -20°C.

DBS Extraction

The extracting solution consisted of $^2H_8$-Rifampicin 0.25 mg/L (internal standard of RIF), cyanoimipramine 0.05 mg/L (internal standard of DAc-RIF, CLR and 14OH-CLR), EDTA 1 g/L and deferoxamine 2 g/L in water. A disc with a diameter of 8 mm was punched out from the central part of DBS and transferred to a 1.5 mL Eppendorf plastic tube. An extracting solution volume of 300 µL was added and the extraction was accelerated by sonication for 20 minutes. After extraction 200 µL of the extract

was transferred to a glass vial and a 600 μL of acetonitrile was added to precipitate the endogenous components in the extract. The sample was vortexed for 1 minute and centrifuged at 11000 rpm for 5 minutes and 5μL of the supernatant was injected into the LC-MS/MS system.

Equipment and Conditions

The LC-MS/MS system was consisted of a Surveyor® MS pump and a Surveyor plus® autosampler connected with a Thermo Fisher Scientific TSQ Quantum Discovery, triple quadrupole mass spectrometry (Waltham, US). The autosampler and column were set at a temperature of 20°C. The chromatographic analysis was performed on a 50 mm×2.1 mm×5μm HyPurity C18 column and (Interscience Breda, the Netherlands). The analytes were eluted with a flow rate of 300 μl/minute using a solvent gradient as followed: 0 – 1 minute, ACN from 0% to 95%, water from 95% to 0%; 1-2.5 minute, ACN 95% and water 0%; 2.5- 2.6 minute, decreased ACN to 0% and kept eluting by 95% water until 3.5 minutes. The aqueous buffer (ammonium acetate 10g/L, acetic acid 35mg/L and trifluoroacetic anhydride 2mg/L) was kept at 5% during the gradient.

The Thermo TSQ Quantum Discovery mass selective detector worked in positive ion mode and performed selected reaction monitoring (SRM) at a scan width of 0.5m/z. The mass parameters for each analytes and the internal standards are presented in Table 1. The ion spray voltage, sheath gas pressure, auxiliary gas pressure and capillary temperature were set at 3500 V, 35 arb (arbitrary unit), 5 arb and 350°C, respectively. The Xcalibur software version 1.4 SR1 was used for peak height integration and quantification (Thermo Fisher, Waltham, USA).

Method development

The DBS at MED level was used for roughly evaluating the efficiency of different methods of extraction. Because the analytical method for simultaneous determination of the analytes in plasma has been successful validated, the DBS analytical method development was focused on the matrix effect and recovery (15). Extracting solvents including water, mixtures of 0%, 30% and 80% acetonitrile in methanol were tested. The extraction was accelerated by a 60 minute of sonication at room temperature and the extract was injected into the LC-MS/MS. The matrix effect and recovery in the method development were evaluated with the sample preparation in triplicate (23). Matrix effects, which may originate from the paper, the extraction solvent and the human blood matrix, were investigated. Similar extraction procedures using neat solution containing analytes in methanol were performed in triplicate for blank paper disc, 10μL of packed red blood cell, 10μL of plasma, 10μL of phosphate buffered saline.

The DBS aqueous extraction suffers from endogenous matrix effects and therefore requires a cleaning process (9,19). A volume of 600 μL acetonitrile was added to precipitate 200 μL DBS aqueous extract containing EDTA and/or deferoxamine. The appropriate concentration of EDTA and deferoxamine in the extracting solution for precipitating the endogenous components was investigated. The extracting solutions with EDTA and deferoxamine at concentrations of 2 g/L were used. The precipitation procedure was performed as described before. UV-VIS absorption spectra with a wavelength range of 200-600nm was obtained from the varying supernatants using Varian UV-VIS spectrometry to indicate the cleanliness of these solutions.

Method validation

The method was validated in accordance with the US Food and Drug Administration's Guidance for Industrial Bioanalytical Method validation (24). The validated criteria included the selectivity, linear-

ity, accuracy and precision, dilution integrity, carry-over, process efficiency and stability. Besides, the influences of hematocrit and blood volume of DBS were evaluated as additional criteria that are recommended for DBS analysis (9,10,23). The validation was performed with maximum tolerated bias and coefficient of variation (CV) of 20% for the lower limit of quantification (LLOQ) and 15% for the other validated concentrations.

Selectivity, specificity and carry-over
The selectivity and specificity were evaluated by comparing the responses of the LLOQ and blank DBS samples prepared (9) from 5 batches of human blood. The average response of blank DBS samples was required to be within 20% of the average response of the LLOQ samples. The carry-over was assessed using the response ratio of a blank sample injected after a HIGH QC and LLOQ. The response was required to be less than 20% of the LLOQ QC.

Linearity, reproducibility and dilution integrity
On each of three consecutive validation days, a single calibration curve was analyzed to assess linearity. The calibration curves were constructed using $1/x^2$ weighted linear regressions. The analytical responses, which were the peak height ratios between analyte and respective internal standard, were used for the quantification. Additionally, in each validation day, five QC´s of LLOQ, LOQ, MED, HIGH and OC (over the calibration curve) were analyzed in five folds to evaluate the intra-day and inter-day accuracy and precision and dilution integrity. The precipitated supernatant from OC samples were diluted 10 times with extract of a blank DBS before injection. Accuracy and the inter-day and intra-day precision was estimated using one-way analysis of variance (one-way ANOVA).

Matrix effect, complexing effect and recovery
The process efficiency was fully evaluated as the matrix effect, complexing effect and recovery at three QC levels of LOW, MED and HIGH. Different solutions A, B, C, D, E, F and G were prepared in five-fold as presented in Table 2. To precisely calculate the theoretical concentration of the neat solution, a blood volume of 10μL was used to prepare the DBS in order to be able for punching the whole DBS for extraction. The prepared solutions were sonicated in 20 minutes and 690 μL of acetonitrile was added to each sample. After vortexing for 1 minute and centrifuging at 11000 rpm in five minutes, the supernatant was transferred to a glass vial and injected into the LC-MS/MS. The influence of Fe (III) and DBS were estimated by assessing the peak height ratios of B/A and C/A, respectively. The recovering effect of adding complexing agents calculated as E/D and F/D. Recovery and process efficiency were G/D and F/D, respectively.

Effect of hematocrit and blood spot volume
Hematocrit and blood spot volume may affect the analytical result and therefore these parameters are evaluated. The effect of Hct was evaluated using QC blood at three Hct values of 20, 35, 50%. The effect of blood spot volume was assessed by preparing DBS with blood volumes of 30, 50 and 100 μL. At each Hct level and blood spot volume, three QC levels of LOW, MED and HIGH were analyzed in five-fold. The analytical results were corrected for their hematocrit value (19). The bias which is calculated as the difference between the analytical result and the nominal concentration (in percentage) present the effect of Hct and blood spot volume.

Stability
The stability of the analytes in DBS was tested with QC levels LOW and HIGH after storing at tem-

peratures of 50°C for 1, 3, 7 and 15 days, at 37°C and ambient temperature for 10, 20 and 30 days and at room temperature for 7, 30 and 60 days. The samples were prepared and analyzed in fivefold and the analytical result was compared with their nominal concentration.

Clinical validation

Adult tuberculosis patients in the TB unit of the Beatrixoord hospital who received rifampicin or clarithromycin were eligible for clinical validation. The proposal was approved by medical ethical committee of University Medical Central Groningen. Informed consents were obtained from all participating patients. Three DBS samples at 0, 2 and 8 hours after dosing for clarithromycin or 1,2 and 4 hours after dosing for rifampicin were collected from each patient by a finger prick to create a bloodspot from a single drop on the paper cards. The DBS samples were then left dry at ambient temperature for 3 hours and stored separately in a sealed plastic bag with a desiccant sachet at -20°C until analysis. A venous blood sample was taken at the same time with DBS sampling using an EDTA vacutainer. After centrifuging for 5 minutes at 3000rpm, the plasma was obtained and stored at -20°C before being routinely analyzed by a validated method (15). The correlation between DBS and plasma analytical results were assessed using ordinary least square regression.

RESULTS

Method development

The DBS extracting method was intensively investigated by testing various extracting solvents. With the use of methanol or its mixture with acetonitrile, a dramatic decrease in peak height of rifampicin was observed. The acetonitrile appeared to negatively influence the recovery. The acetonitrile proportion up to 84% in methanol resulted in recovery of less than 3% for all four analytes (Table 3).

During method development it was observed that the rifampicin peak height was negatively influenced by matrix effects of DBS and red blood cells. The same effect was observed if $FeCl_3$ 50mg/L was added to the neat solution. The propose assumption is that rifampicin may bind with components in the DBS matrix in which Fe^{3+} was suggested to be a potential factor (20). Therefore, the un-fragmented mass of RIF-Fe(III) (m/z= 879, collision energy = 0eV) was checked and a small peak at the identical retention time with rifampicin was observed. The efforts to adjust the chromatographic conditions or by adding complexing agents (i.e. EDTA and DFX) were however unsuccessful to recover the response of rifampicin.

The aqueous extraction of DBS yield a dark red extract which is not suitable to directly inject into LC-MS/MS system. Using acetonitrile and methanol did not sufficiently precipitate the endogenous components in the extract. However, the use of an aqueous extracting solution containing EDTA can initiate the precipitation of endogenous components. With the EDTA concentration range from 0 to 1.58 g/L, the precipitation was dependent on the EDTA concentration. As the concentration of EDTA increased, the amount of precipitating endogenous components in the DBS was increased and therefore the supernatant was cleaner. At EDTA concentration higher than 0.58 g/L, the precipitated extracts appeared to be totally colorless and UV-VIS spectra presented no significant absorbance peak (Figure 1). Although the EDTA concentration of 0.58 g/L was sufficient to precipitate endogenous components in the DBS, the EDTA concentration of 1 g/L was used as between patient variability in blood characteristics may occur. The precipitation of endogenous components from the aqueous extract of DBS using EDTA and acetonitrile was simple, rapid and can be applied to analysis method for other drugs also.

Although the precipitated extraction was optically clean with the presence of EDTA, the peak height of rifampicin was still approximately 50% of the peak height of respective neat solution. It was recovered to approximately 100% after adding deferoxamine 1mg/L to the extracting solution.

Method validation

Selectivity, specificity and carry over

The mean response of the blank samples accounted for less than 4.6% of the response of LLOQ samples. In addition, all six batches of human blood showed no signal higher than 20% response of the LLOQ sample prepared from the same matrix. A corresponding chromatogram is presented in figure 2. No carry-over was observed for all four analytes as the responses of the blank sample after injecting a HIGH QC sample were less than 20% response of LLOQ samples.

Linearity, reproducibility and dilution integrity

The method showed to be linear with correlation coefficients (r^2) between the calibration curves of RIF, DAc-RIF, CLR and 14OH-CLR of 0.9953, 0.9971, 0.9987 and 0.9986, respectively. The linear model test based on ANOVA showed no significant lack of fit. The CV and bias (n=3) at each calibration level were all less than 15%.

The reproducibility of the method was evaluated as the accuracy and the within day and between day precision. The accuracy and precision estimated by one-way ANOVA analysis were tolerated according to the criteria of the FDA guidelines in which acceptable bias and CV are less than 20% for LLOQ and 15% for other validation concentrations (Table 3).

The accuracy and precision results for the 10 times diluted OC samples were within the acceptance of FDA guidance and proved the dilution integrity of the method (Table 3).

Matrix effect, complexing effect and recovery

There was a dramatic drop of the peak height of rifampicin in the presence of $FeCl_3$ or DBS matrix. With the use of an extracting solution containing complexing agents, the effects of both matrices on the response of rifampicin were neutralized. The matrix effects of DBS with the presence of EDTA and DFA were 102%, 106% and 96% for the LOW, MED and HIGH levels of RIF respectively. The peak height of DAc-RIF showed a small increase with the use of the complexing agents. The matrix effects of CLR and 14OH-CLR were of minor extent regardless the presence of the complex agents. A high recovery of 88% to 102% was achieved at three QC levels for CLR and 14OH-CLR. A lower recovery of RIF and Dac-RIF was observed yielding lower process efficiencies. Nevertheless, comparing to extraction by methanol or its mixture with acetonitrile the recovery of aqueous extraction is much improved (Table 4).

Effect of hematocrit and blood spot volume

The biases caused at Hct ranges from 20 to 50% were within the acceptance limit of 15% for CLR and 14OH-CLR at all three QC level. For RIF and DAc-RIF, however, a clear upward trend in bias was observed as Hct increased, resulting in a bias range from -18.4 % to 28.0 %. Correcting with the respective hematocrit value, the upward bias trend was less steep, ranging from -8.9% to 15.8%.

The bias range due to the variation in blood spot volume was from -7.8% to 8.9% for all four analytes at QC MED and HIGH. At LOW QC the blood spot volume of 30 μL resulted in negative biases as low as -23.6, -19.4, -17.6 and -20.6 % for RIF, DAc-RIF, CLR and 14OH-CLR, respectively.

Stability

A high stability was observed with CLR and 14OH-CLR in DBS in which no significant degradation occurred at 50°C, 30°C and at room temperature for 15 days, 30 days and 2 months, respectively. RIF and DAc-RIF showed stable at room temperature for up to two months. At higher temperature of 30°C and 50°C, RIF and DAc-RIF were stable for 10 days and for 3 days, respectively. Longer storage at temperatures over 30°C resulted in more than 15% degradation.

Clinical validation

Thirteen patients receiving rifampicin agreed to join the study. One patient experienced vomiting on the study day and was excluded. From the remaining twelve patients, eight pairs had RIF's concentrations under LLOQ (0.2mg/L for plasma and 0.15mg/L for DBS) both in plasma and in DBS. The correlation coefficient between RIF's concentration in DBS and plasma was 0.9067 and the slope of the regression line was 0.86 (n=28). The DAc-RIF concentrations lower than LLOQ (0.2mg/L for plasma and 0.15mg/L for DBS) were observed in 16 pairs of samples. Two pairs of samples with DAc-RIF concentration above LLOQ for DBS (0.19mg/L and 0.23mg/L) yet below LLOQ for plasma were not included in regression. The correlation coefficient between DBS and plasma was 0.6856 (n=18) for DAc-RIF. Four MDR-TB patients treated with clarithromycin participated to the study and 12 pairs of DBS-plasma samples were taken. CLR and 14OH-CLR concentration were under LLOQ level in two pair of samples (0.1mg/L for plasma and 0.05 mg/L for DBS). Good correlations between DBS and plasma samples of CLR and 14OH-CLR were observed (r^2=0.9752 for CLR and r^2=0.9421for 14OH-CLR). The slope of the regression line of CLR was 0.7964 which was lower than 1 suggesting a higher concentration in plasma than in DBS. The metabolite of CLR showed an equal result between DBS and plasma (Figure 3).

DISCUSSION

This study showed that RIF, CLR and their active metabolites can be measured in DBS and show a good correlation with plasma. Therefore, method is suitable for therapeutic drug monitoring and pharmacokinetic studies. The analytical method is validated and the DBS specimen was proved to be very stable for a long period. In addition, a simple extraction procedure was developed to deal with the interaction of the analytes with the DBS matrix.

The challenge of developing a simple extraction method was to cope with low recoveries and/or substantial matrix effects for RIF and its metabolites observed after using acetonitrile or methanol or their mixtures as extraction solvents. As a consequence, the pre-validation experiment showed non-linear calibration curves and irreproducible results. The matrix effect was resulted from the blood matrix of DBS and whole blood. The presumed forming of the complex between RIF and endogenous components in blood extract is a previous suggested theory (20,22). The formed complex was unstable and showed to have a slightly different retention time, while the complex showed in-source fragmentation back into the analyte during ionization. This caused a shoulder following the analyte peak and thus deteriorating the chromatographic quality of the analysis and lowering the peak height at the RIF's retention time. The use of complexing agents such as EDTA and DFX to eliminate complex forming with endogenous substances during the extraction was unsuccessful for methanol and acetonitrile extracts. During method development it was observed that an aqueous extraction provided an appropriate environment for interaction between the DBS matrix and complexing agents. EDTA showed to improve the precipitation of the DBS extract yielding a cleaner supernatant. Without EDTA, the dirty extract produced a negative effect on the response of RIF and DAc-RIF but no such clear effect

was observed with CLR and 14OH-CLR. Interestingly, the precipitation appeared to be dependent on EDTA concentration. An EDTA concentration higher than 0.58 g/L showed sufficient for precipitation. The incorporation of EDTA recovered the response of the neat RIF's solution containing Fe (III) but not of the DBS extract. It was supposed that RIF may interact with other endogenous components in DBS rather than Fe (III) only. It was reported that DFX as a ferric chelating agent improves the analytical response of artemisinin derivatives (25). In this study, adding DFX to the extraction solution recovers the response of RIF in the DBS extraction to approximately 100% (Table 3).

The DBS sampling without a volumetric device like a pipette is confronted with an analytical bias resulting from variable Hct or blood volume of the spot. As Hct values increased higher concentrations were measured for the same concentration level for all four analytes. A significant bias occurred with Hct values extremely distant from the standardized value, Correction of the analytical result for its Hct value reduces this bias to an acceptable range (19). Biases of approximately 20% were observed with the blood spot of 30mL at LOW QC concentration. This may result from a chromatographic effect in which the analytes stay more in the centre part of the DBS. The use of a volumetric capillary may be considered to reduce this kind of bias (10,23).

The clinical validation in our study showed a good correlation between DBS and plasma concentrations for CLR, RIF and 14OH-CLR. The results of DBS as a consequence can be used to predict the plasma concentration which is well investigated in pharmacokinetics study. The correlation coefficient for DAc-RIF was low (r^2=0.69) that may result in error in predicting plasma concentration from DBS concentration. Because concentration of Dac-RIF is much lower than respective concentration of RIF, the TDM decision was affected at minor extent due to such error. It is noticeable that sample under LLOQ levels of DBS and plasma samples was 100% matched for CLR, RIF and 14OH-CLR and 89% matched for DAc-RIF. This result at LLOQ level suggests that DBS sampling can also be useful to monitor the adherence of patients.

One of the most attractive issues of DBS in bioanalysis is the stability. This study demonstrates that RIF and CLR and metabolites are stable up to 2 months under the room condition with the temperature of approximately 25°C. All four substances also showed to be stable at 37°C, which mimics a tropical climate and is common in the high burden areas like South Africa and Asia. In these conditions, CLR and its metabolite was not affected after 2 months whilst RIF and DAc-RIF were stable after 10 days of storage only. CLR and it metabolites also presented a high stability at high temperature up to 50°C as no significant degradation was observed after 15 days. RIF and its metabolite were considered to be stable after 3 days of storage in that condition only. Although the stability of RIF and DAc-RIF was less than CLR, 10 days at 37°C or 3 days at 50°C could be enough for transporting sample even by normal post.

In conclusion, the rapid LC-MS/MS method was developed and thoroughly validated to simultaneously quantify RIF, CLR and their metabolites in dried blood spot. The role of EDTA and DFX as a chelating agent in the extraction was well investigated and suggests a potential application for other DBS analytical methods. The clinical validation showed a good correlation between the result of DBS and conventional sampling. The long term stability of the DBS specimen at high temperature could facilitate the TDM and pharmacokinetic studies of RIF and CLR even in resource limited areas.

REFERENCES

(1) Nienhuis WA, Stienstra Y, Thompson WA, Awuah PC, Abass KM, Tuah W, et al. Antimicrobial treatment for early, limited Mycobacterium ulcerans infection: a randomised controlled trial. Lancet 2010 Feb 20;375(9715):664-672.

(2) van Crevel R, Alisjahbana B, de Lange WCM, Borst F, Danusantoso H, van der Meer JWM, et al. Low plasma concentrations of rifampicin in tuberculosis patients in Indonesia. Int J Tuberc Lung Dis 2002 JUN;6(6):497-502.

(3) Wilkins JJ, Savic RM, Karlsson MO, Langdon G, McIlleron H, Pillai G, et al. Population pharmacokinetics of rifampin in pulmonary tuberculosis patients, including a semimechanistic model to describe variable absorption. Antimicrob Agents Chemother 2008 Jun;52(6):2138-2148.

(4) WALLACE R, BROWN B, GRIFFITH D, GIRARD W, TANAKA K. Reduced Serum Levels of Clarithromycin in Patients Treated with Multidrug Regimens Including Rifampin Or Rifabutin for Mycobacterium-Avium Mycobacterium-Intracellulare Infection. J Infect Dis 1995 MAR;171(3):747-750.

(5) Alffenaar JW, Nienhuis WA, de Velde F, Zuur AT, Wessels AM, Almeida D, et al. Pharmacokinetics of rifampin and clarithromycin in patients treated for Mycobacterium ulcerans infection. Antimicrob Agents Chemother 2010 Sep;54(9):3878-3883.

(6) Rodvold K. Clinical pharmacokinetics of clarithromycin. Clin Pharmacokinet 1999 NOV;37(5):385-398.

(7) ACOCELLA G. Clinical Pharmacokinetics of Rifampicin. Clin Pharmacokinet 1978;3(2):108-127.

(8) van Ingen J, Egelund EF, Levin A, Totten SE, Boeree MJ, Mouton JW, et al. The Pharmacokinetics and Pharmacodynamics of Pulmonary Mycobacterium Avium Complex Disease Treatment. Am J Respir Crit Care Med 2012 Jun 28.

(9) Vu DH, Alffenaar JW, Edelbroek PM, Brouwers JR, Uges DR. Dried blood spots: a new tool for tuberculosis treatment optimization. Curr Pharm Des 2011;17(27):2931-2939.

(10) Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther Drug Monit 2009 06;31(3):327-336.

(11) Srivastava A, Waterhouse D, Ardrey A, Ward SA. Quantification of rifampicin in human plasma and cerebrospinal fluid by a highly sensitive and rapid liquid chromatographic-tandem mass spectrometric method. J Pharm Biomed Anal 2012 Nov;70:523-528.

(12) Calleja I, Blanco-Prieto MJ, Ruz N, Renedo MJ, Dios-Vieitez MC. High-performance liquid-chromatographic determination of rifampicin in plasma and tissues. J Chromatogr A 2004 Mar 26;1031(1-2):289-294.

(13) Allanson AL, Cotton MM, Tettey JN, Boyter AC. Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: a potential method for therapeutic drug monitoring. J Pharm Biomed Anal 2007 08/15;44(0731-7085; 4):963-969.

(14) Oswald S, Peters J, Venner M, Siegmund W. LC-MS/MS method for the simultaneous determination of clarithromycin, rifampicin and their main metabolites in horse plasma, epithelial lining fluid and bronchoalveolar cells. J Pharm Biomed Anal 2011 Apr 28;55(1):194-201.

(15) de Velde F, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2009 Jun 15;877(18-19):1771-1777.

(16) Gitau EN, Muchohi SN, Ogutu BR, Githiga IM, Kokwaro GO. Selective and sensitive liquid chromatographic assay of amodiaquine and desethylamodiaquine in whole blood spotted on filter paper. J Chromatogr B Analyt Technol Biomed Life Sci 2004 01/05;799(1570-0232; 1):173-177.

(17) Koal T, Burhenne H, Romling R, Svoboda M, Resch K, Kaever V. Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2005;19(0951-4198; 21):2995-3001.

(18) Barfield M, Spooner N, Lad R, Parry S, Fowles S. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies. J Chromatogr B Analyt Technol Biomed Life Sci 2008 07/01;870(1570-0232; 1):32-37.

(19) Vu DH, Koster RA, Alffenaar JW, Brouwers JR, Uges DR. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. J Chromatogr B Analyt Technol Biomed Life Sci 2011 May 1;879(15-16):1063-1070.

(20) Ke J, Yancey M, Zhang S, Lowes S, Henion J. Quantitative liquid chromatographic-tandem mass spectrometric determination of reserpine in FVB/N mouse plasma using a "chelating" agent (disodium EDTA) for releasing protein-bound analytes during 96-well liquid-liquid extraction. J Chromatogr B Biomed Sci Appl 2000 Jun 9;742(2):369-380.

(21) Fujimoto T, Tsuda Y, Tawa R, Hirose S. Fluorescence polarization immunoassay of gentamicin or netilmicin in blood spotted on filter paper. Clin Chem 1989 05;35(0009-9147; 5):867-869.

(22) Ascenzi P, Bolli A, di Masi A, Tundo GR, Fanali G, Coletta M, et al. Isoniazid and rifampicin inhibit allosterically heme binding to albumin and peroxynitrite isomerization by heme-albumin. J Biol Inorg Chem 2011 Jan;16(1):97-108.

(23) Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed Chromatogr 2010 01;24(1099-0801; 0269-3879; 1):49-65.

(24) US Department of Health and Human Services Food and Drug Administration. Guidance for Industry. Bioanalytical Method Validation. ; 2001.

(25) Lindegardh N, Hanpithakpong W, Kamanikom B, Pattayaso J, Singhasivanon P, White NJ, et al. Quantification of dihydroartemisinin, artesunate and artemisinin in human blood: overcoming the technical challenge of protecting the peroxide bridge. Bioanalysis 2011 Jul;3(14):1613-1624.

Table 1. The mass condition and the concentration of calibration and quality control sample

Component	Mass transition (m/z)		CE (eV)	Calibration concentration (mg/L)	QC sample concentration (mg/L)				
	Parent	Product			LLOQ	LOW	MED	HIGH	OC
Clarithromycin	748.5	590.2	18	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.05	0.15	5.00	8.00	20.00
14-Hydroxy clarithromycin	764.4	606.2	20	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.05	0.15	5.00	8.00	20.00
Rifampicin	823.3	791.2	17	0.15, 0.45, 1.5, 3.0, 9.0, 15.0, 24.0, 30.0	0.15	0.45	15.00	24.00	60.00
Deacetyl rifampicin	781.4	749.2	14	0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.15	0.50	5.00	8.00	20.00
2H8-Rifampicin	831.5	799.5	17	-	-	-	-	-	
Cyanoimipramine	306.2	218.0	39	-	-	-	-	-	

CE: collision energy

Table 2. Experimental design to investigate the matrix effect, complexing effect, recovery and process efficiency

Components	Solution									
	A	B	C	D	E	F	G	H	I	K
Neat solution (ml)	200	200	200	200	200	200	200	200	200	
$FeCl_3$ 1g/L (ml)		10			10			10		
EDTA 23g/L (ml)				10	10	10	10	10	10	10
DFX 23g/L (ml)							10	10	10	10
Blank DBS (disc)			1			1			1	
QC DBS (disc)										1
Water (ml)	30	20	30				10		10	210

Solutions were prepared in water; Neat solution: containing analytes at theoretical concentrations of LOW, MED and HIGH level.

Table 3. Effect of different extracting solution on the matrix effect and recovery

	Extracting solution (ACN: MeOH)	14OH-CLR	CLR	DAc-RIF	RIF
Matrix effect (%)	0:100	109	106	63	15
	30:70	113	112	74	19
	80:20	103	103	59	17
Recovery (%)	0:100	68	75	54	40
	30:70	59	65	44	32
	80:20	2	3	1	2

Data calculated from 3 replications

Table 4. Accuracy, precision and the diluent integrity (n=5)

	CLR					14OH-CLR					RIF					Dac-RIF				
	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC
Nominal concentration (mg/L)	0.05	0.15	5.0	8.0	20.0	0.05	0.15	5.0	8.0	20.0	0.15	0.45	15.0	24.0	60.0	0.15	0.5	5.0	8.0	20.0
Accuracy (% bias)	0.3	10.5	3.6	0.2	-4.0	-3.9	4.7	4.7	2.7	-7	-1.1	1.9	1.6	-0.5	4.0	0.1	5.0	1.4	-0.1	12
Within-run precision (% CV)	9.1	3.7	2.6	2.3	3.2	10.7	5.6	3.4	3.3	3.2	5.4	2.1	3.2	2.7	3.8	12.5	6.0	4.8	4.2	4.1
Between run precision (% CV)	0.0	4.8	0.6	0.0	5.9	6.4	7.9	3.7	0.0	6.2	7.2	2.6	2.4	3.7	6.2	11.0	4.8	0.0	0.0	7.1
Overall precision (%CV)	9.1	6.0	2.7	2.3	6.7	12.5	9.7	5.0	3.3	7.0	9.0	3.3	4.0	4.6	7.3	16.6	7.7	4.8	4.2	8.2

OC: Over the calibration curve concentration (diluted 10 times).

Table 5. Matrix effect, complexing effect, recovery and process efficiency (n=5)

		Response ratio	RIF			DacRIF			14OH CLR			CLR		
			LOW	MED	HIGH	LOW	MED	HIGH	LOW	MED	HIGH	LOW	MED	HIGH
Matrix	Complexing agents													
Fe (III)	-	B/A	28%	17%	17%	52%	48%	51%	106%	101%	102%	105%	115%	115%
DBS	-	C/A	60%	34%	28%	68%	53%	50%	85%	93%	91%	98%	106%	101%
Fe (III)	EDTA	E/D	68%	96%	91%	42%	67%	86%	96%	104%	106%	117%	110%	108%
DBS	EDTA	F/D	42%	42%	37%	41%	38%	51%	78%	96%	98%	106%	114%	111%
Fe (III)	EDTA,DFX	H/G	97%	108%	91%	69%	87%	96%	105%	107%	104%	109%	110%	103%
DBS	EDTA,DFX	I/G	102%	106%	96%	67%	78%	89%	85%	97%	99%	100%	103%	102%
Recovery		K/I	70%	91%	87%	70%	85%	82%	88%	92%	90%	99%	102%	95%
Process efficiency		K/G	71%	96%	83%	47%	66%	73%	75%	89%	90%	99%	105%	98%

Data is presented as % calculated from ratio means of five replications.

Table 6. Long term stability under different storage temperature (n=5)

Temperature	Time	CLR		14OH CLR		RIF		DacRIF	
	(days)	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW
25°C	7	2.4 (3.8)	4.8 (7.7)	4.3 (3.3)	9.2 (9.7)	-0.6 (3.4)	5.5 (4.3)	1.1 (5.9)	1.2 (12.7)
	30	0.1 (3.8)	9.2 (3.8)	2.1 (5.0)	4.5 (5.2)	-1.7 (2.6)	-2.6 (5.2)	-0.6 (5.7)	2.4 (3.2)
	60	-5.5 (1.8)	7.8 (4.8)	-3.0 (3.9)	3.0 (8.9)	-14.5 (2.2)	-4.3 (2.4)	-11.5 (5.0)	-3.3 (4.5)
37°C	10	-7.0 (3.2)	10.7 (6.3)	-0.6 (1.5)	1.4 (3.3)	-14.1 (3.4)	0.6 (4.1)	-11.1 (1.7)	-4.8 (5.8)
	20	-7.7 (4.8)	8.0 (6.1)	-6.4 (4.5)	-1.7 (4.5)	-19.2 (1.7)	-8.9 (5.8)	-20.3 (2.5)	-8.0 (7.1)
	30	-6.7 (3.4)	13.9 (6.5)	-4.4 (3.4)	-0.2 (5.7)	-28.2 (5.3)	-15.0 (6.8)	-26.4 (4.0)	-15.7 (8.9)
50°C	1	-0.8 (2.6)	8.2 (4.1)	3.6 (1.4)	1.5 (4.9)	0.8 (2.6)	-1.2 (6.0)	3.2 (3.7)	5.8 (6.4)
	3	3.9 (1.2)	-0.7 (4.1)	8.8 (2.8)	5.7 (5.2)	-9.1 (2.1)	-9.8 (4.7)	-5.5 (5.0)	-11.9 (7.3)
	7	0.0 (5.0)	2.0 (2.8)	6.0 (7.0)	5.0 (2.6)	-19.0 (5.5)	-15.7 (4.5)	-14.0 (5.3)	-18.4 (10.1)
	15	-1.9 (3.8)	9.4 (4.4)	-1.1 (3.0)	-6.9 (6.4)	-24.2 (5.4)	-20.2 (5.0)	-25.8 (7.0)	-31.3 (12.2)

Result was presented as %Bias (%CV) of five replications

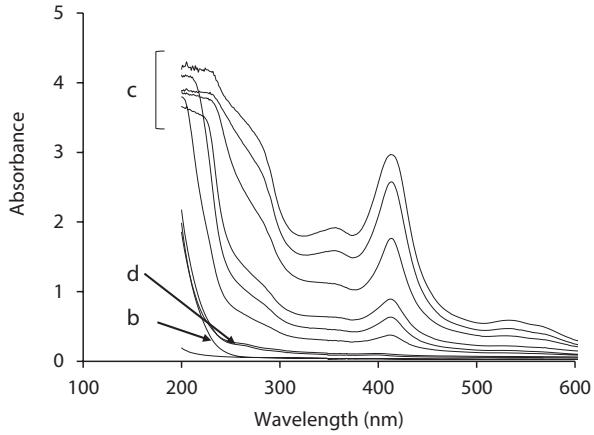

Figure 1. UV-VIS absorbance of the precipitated DBS extracts using different EDTA concentration.

a: ACN: water (v/v 3:1); b: Blank extraction using EDTA 1.54g/L in water; c: Extract of DBS using EDTA concentration of 0-0.38 mg/L in water; d: extract of DBS using EDTA concentration of 0.58-1.54 g/L in water;

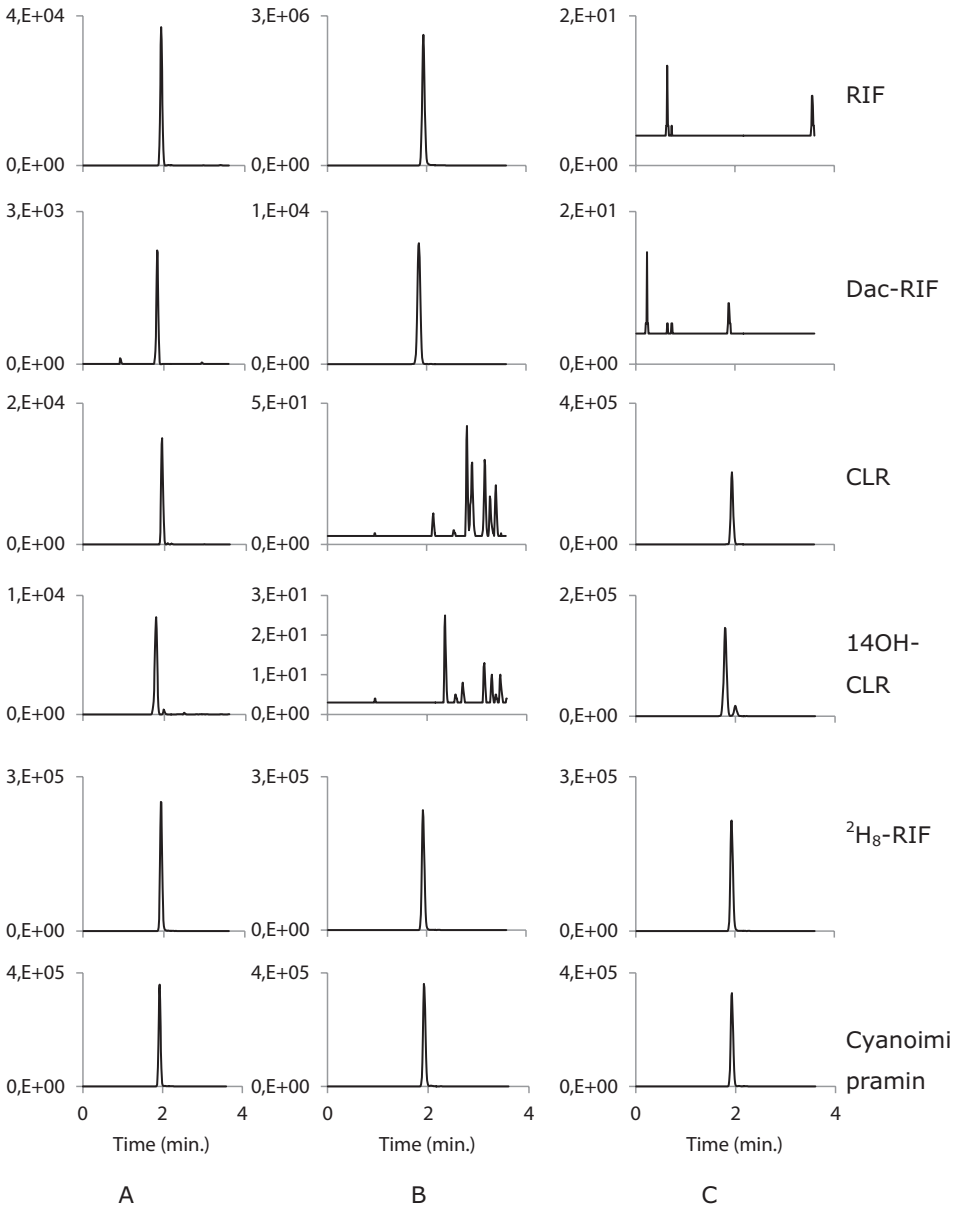

Figure 2.

Representing chromatograph; A: LLOQ DBS sample; B: DBS sample of a TB patient at t=2h after administered rifampicin (DBS concentration RIF=11.00 mg/L, Dac-RIF=0.51 mg/L); C: B: DBS sample of a TB patient at t=2h after administered clarithromycin (DBS concentration CLR=0.80mg/L, 14OH-CLR = 0.92mg/L).

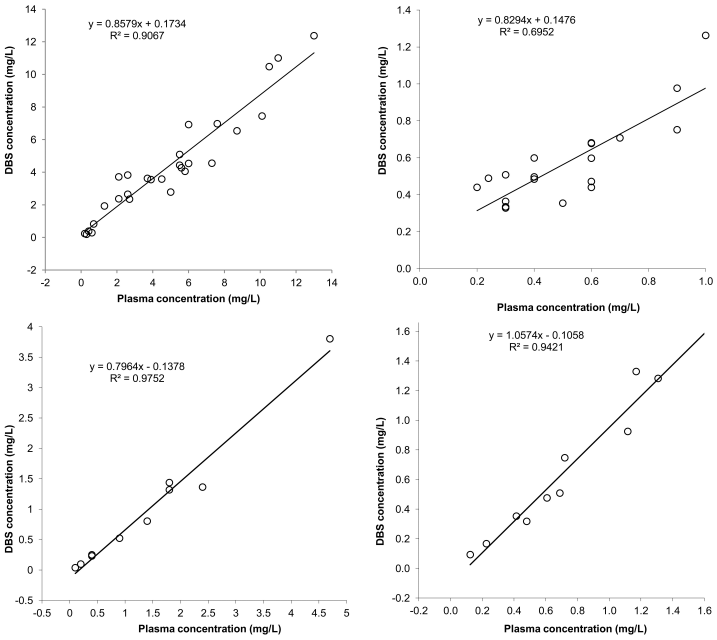

Figure 3. Correlation between the DBS and plasma concentration

Uper-lelf: Rifampicin (n=28); Uper-right: Desacetyl Rifampicin (n=18)

Lower-left: Clarithromycin (n=10); Lower-right: 14 Hydroxy clarithromycin (n=10)

Chapter

7

Dried Blood Spot Analysis for Therapeutic Drug Monitoring of Linezolid in Patients with Multidrug –Resistant Tuberculosis

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Dried blood spot analysis for therapeutic drug monitoring of linezolid in patients with multidrug-resistant tuberculosis

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ABSTRACT

Linezolid is a promising antimicrobial agent for the treatment of multidrug-resistant tuberculosis (MDR-TB), but its use is limited by toxicity. Therapeutic drug monitoring (TDM) may help to minimize toxicity whilst adequate drug exposure is maintained. Conventional plasma sampling and monitoring might be hindered by logistic problems in most parts of the world that may be solved by dried blood sampling (DBS). The aim of this study is to develop and validate a novel method for TDM of linezolid in MDR-TB patients using DBS.

Plasma, venous DBS and capillary DBS specimens were obtained simultaneously from eight patients receiving linezolid. A DBS method was developed and clinically validated by comparing DBS with plasma results using Passing Bablok regression and Bland-Altman analysis.

This study showed that DBS analysis was reproducible and robust. Accuracy and between and within-day precision from three validation presented as bias and CV were less than 17.2% for lower limit of quantification level (LLOQ) and 7.8% for other levels. The method showed a high recovery of approximately 95% and a low matrix-effect of less than 8.7%. DBS specimens were stable at 37°C for 2 months and at 50°C for one week. The concentration ratio of DBS/plasma was 1.2 (95%CI: 1.12-1.27). Linezolid exposure calculated from DBS and plasma showed good agreement.

In conclusion, DBS analysis of linezolid is a promising tool to optimize linezolid treatment in MDR-TB patients. An easy sampling procedure and high sample stability may facilitate TDM, even in underdeveloped countries with limited resources where conventional plasma sampling is not feasible.

INTRODUCTION

Linezolid is used as a second line drug in the treatment of multidrug-resistant tuberculosis (MDR-TB) due to its efficacy *in vitro* (21), *in vivo* (9) and in patients (1, 2, 14, 17, 34) against *Mycobacterium tuberculosis*. The World Health Organization (WHO) classifies linezolid as a reserve anti-tuberculosis drug for the treatment of multidrug-resistant/extensively drug-resistant tuberculosis (MDR/XDR-TB) (33). Linezolid is usually added to a treatment regimen consisting of anti-tuberculosis drugs for which the *Mycobacterium tuberculosis* is still susceptible. However, treatment with linezolid may be limited by toxicity, such as time- and dose-dependent neuropathy or myelosuppression (17, 29), urging dose reduction or cessation of treatment with linezolid. Therapeutic drug monitoring (TDM) can be used to implement dose reductions to limit toxicity, whilst preventing inadequate exposure. Efficacy predicting pharmacokinetic /pharmacodynamic (PK/PD) parameters, such as the area under the concentration-time curve to MIC ratio ($AUC_{0\text{-}24h}$/MIC), might be helpful in evaluating linezolid dosages (1, 7, 26, 32) . The $AUC_{0\text{-}24h}$/MIC has been shown to be the best predictive model in a murine model (32), but evidence from human data are lacking. Further PK/PD data from TB-programs or large studies are needed for the development of evidence based PK/PD parameters, such as an $AUC_{0\text{-}24h}$/MIC ratio target.

Linezolid treatment has been evaluated for TB treatment, in several case series (17, 23). However, neither drug susceptibility testing (DST) nor drug exposure assessment was performed for linezolid, making it difficult to draw conclusions on efficacy (5). For instance, drug-interactions with other antimicrobial agents might have occurred and may have had an impact on linezolid exposure (6, 15). In addition, conventional drug exposure evaluation for TB drugs using plasma samples might have been hindered in these studies by logistical challenges (30). The use of dried blood spot (DBS) sampling may provide a helpful alternative to conventional plasma sampling through simplified sampling procedure and increased sample stability. DBS sampling has been applied in the treatment of other infectious diseases like malaria and HIV (30). Other advantages may include a lower required blood sample volume and lower biohazard risk of DBS samples compared to conventional plasma samples (12, 18, 30). Compared to conventional sampling, DBS sampling may be hindered by inter and intra-patient hematocrit (Hct) variation causing different blood viscosity yielding a proportional analytical bias with Hct value. Furthermore, Hct may affect the drug blood / plasma partition ratio complicating the comparison with conventional plasma samples. In the development of a bioanalytical method for linezolid using DBS analysis important patient related factors like blood spot volume, Hct value (3, 24) and difference between capillary and venous blood, have to be assessed during validation. (12, 18, 25, 30). To enable individualized linezolid treatment the aim of this study was to develop and validate a method for DBS analysis and evaluate it in MDR-TB and XDR-TB patients.

MATERIALS AND METHODS

Patients

From September 2010 to March 2012, MDR-TB patients (≥18 years) were recruited from the Tuberculosis Centre Beatrixoord, University Medical Center Groningen (Haren, The Netherlands). Eligible for inclusion were patients receiving treatment with anti-tuberculosis drugs for which routine therapeutic drug monitoring was scheduled. Patients with bleeding disorders were excluded from the study. The study procedures were reviewed and approved by the local Ethics Committee. Patients receiving linezolid were included after providing written informed consent.

Sampling was performed at least one week after the start of linezolid treatment to ensure the steady-state was achieved. Venous blood samples were obtained our before drug intake and at 1, 2, 3, 4 and 8 hours after dosing according to a previous study (2) and local procedures for TDM of TB drugs to be able to calculate drug exposure and other PK parameters. Venous dried blood spot (VDBS) specimens were prepared by pipetting 50μL venous blood onto Whatman 31 ET CHR paper. The remaining venous blood was centrifuged at 3000 rpm for 5 minutes at room temperature to attain plasma which was stored at -20°C until analysis. DBS specimens were obtained through a finger prick by dropping the blood directly on dried blood spot paper. DBS samples were obtained before drug intake, 2 and 8 hours after dosing, representing low, high and medium linezolid blood levels respectively. Both the VDBS and DBS samples were left to dry at room temperature and stored in sealed plastic bags with desiccant sachets at -20°C until analysis.

DBS analysis

To quantify DBS samples an 8 mm-diameter disc was punched out of each blood spot. Extraction of these discs was performed by sonication with a frequency of 47 kHz during a period of 20 minutes using 500 μL of extracting solvent consisting of cyanoimipramine 0.3 mg/L (internal standard) and EDTA 1g/L in water. From this solution, a volume of 200 μL was added to 750 μL of acetonitrile. The samples were vortexed for 1 minute and subsequently centrifuged at 11000 rpm for 5 minutes. An injection volume of 5 μL was analyzed using a validated LC-MS/MS analysis method (16). The plasma samples were prepared and analyzed using the same method.

DBS analytical method validation

The DBS analytical method was validated in accordance with the recommendation of US Food and Drug Administration's (FDA) Guidance for Industry Bioanalytical Method validation (27). For the validation, blood was prepared by mixing plasma, red blood cell and linezolid stock solution to achieve blood at desire concentration and Hct. Subsequently, the validation DBS samples were prepared by pipetting 50μL of blood onto the paper. Linearity was assessed with $1/x^2$ weighting over a concentration range of 0.05-40 mg/L. Clinical relevant concentrations were well within the range of the assay standards (2). The within-day and between-day accuracy and precision were evaluated on four validation levels of LLOQ (lower limit of quantification), LOW, MED and HIGH at concentrations of 0.05, 0.25, 15 and 30 mg/L, respectively. Each validation level was analyzed in fivefold on three consecutive days. The matrix effect and the recovery of linezolid from DBS were determined using a common method (18, 31). The stability of DBS specimens was assessed by storing validation DBS at ambient condition and 37°C after one week, two weeks and two months. As a worst case scenario the stability of DBS specimens was also assessed at 50°C after one day, two days and one week. The stability was evaluated at LOW and HIGH levels in fivefold by comparing the analytical result with the nominal concentrations. In addition to the criteria suggested in the FDA guideline (27), the impact on assay accuracy and precision due to the variations of Hct and blood spot volume were evaluated. For these purposes, Hct of 20, 25, 30, 35, 40, 45 and 50%, and blood spot volumes of 30, 50, 70 and 90 μL were assessed. During the method validation, blood spot volume and Hct were standardized at 50μL and 35%, respectively. The set of Hct of 35% reflects the Hct in tuberculosis patients (3).

Pharmacokinetic and pharmacodynamic evaluation

Pharmacokinetic parameters were evaluated using a non-compartmental model of the KINFIT module of MW Pharm (version 3.9; Mediware, The Netherlands). The $AUC_{0\text{-}12h}$ was calculated using the trapezoidal rule from 0 up to 12 hours and the $AUC_{0\text{-}24h}$ by doubling the $AUC_{0\text{-}12h}$. The maximum concentra-

tion (C_{max}) was defined as the highest observed linezolid concentration with T_{max} as corresponding time. The elimination half-life ($t_{1/2}$) was calculated by dividing the natural logarithm of 2 (ln2) by the elimination constant (k_e) as calculated by MW Pharm. The apparent clearance (Cl) of linezolid was calculated by dose/AUC_{0-12h}. The volume of distribution (V_d) was calculated by dividing Cl with k_e.

The drug susceptibility testing of the *Mycobacterium tuberculosis* isolates was performed at the Dutch National Mycobacteria Reference Laboratory (National Institute for Public Health and the Environment; RIVM) using the Middlebrook 7H10 agar dilution method (28). The AUC_{0-24h}/MIC ratio, often used as a predictive pharmacodynamic parameter for efficacy, was calculated (32).

Statistics

In the method validation, the bias was defined as the difference (in percentage) between analytical result and the nominal concentration. The method was clinically validated by comparing the concentrations of DBS and VDBS with plasma concentrations using Passing Bablok regressions and Bland-Altman analysis by applying the software tool Analyse-it 2.20° (Analyse-it Software, Ltd). Conversion factors, calculated from geometric mean (V)DBS/plasma concentration ratios, were used to calculate conversed DBS and VDBS concentrations(4). Subsequently, the conversed concentrations were used to calculate the AUC_{0-12h} of DBS and VDBS. The agreement between AUC_{0-12h} value of conversed DBS and plasma was evaluated using Bland Altman analysis. Spearman correlation and Wilcoxon signed-rank test was applied to other comparisons.

RESULTS

Patients

Eight patients with a median age of 29 years (IQR: 24-33 years) were included in this study. The baseline characteristics are presented in Table 1. The median of Hct was 37.4% (IQR: 33.0-41.4%). At time of the study three of eight patients received linezolid 300 mg twice a day and five patients in a dose of 600 mg twice daily. Isolates of seven patients showed resistance to first-line drugs isoniazide, rifampicin, ethambutol, pyrazinamide, and streptomycin. The isolate of one patient showed resistance to all first-line drugs except pyrazinamide. All DSTs revealed resistance for rifabutin, whereas one isolate showed fluoroquinolone-resistance and three protionamide-resistances. None of the patients experienced significant discomfort from the finger pricks during DBS sampling which was supported by the fact that all completed the three consecutive samples in this study.

DBS method validation

The DBS assay method showed linearity over the analytical concentration range. The pooled correlation coefficient was r^2=0.9947. The regression equation is: concentration= (0.1635±0.0025) response + (0.0001±0.0003). Within-day and between-day accuracy and precision showed CVs within accepted range. Within-day CVs ranged from 1.6% to 13.8% and between-day CVs from 3.5% to 10.2%. The mean measured concentration was within 98.7% to 106.3% of the nominal concentration. The bias caused by variable matrices, i.e. DBS and EDTA matrices, was less than 8.7%. The recovery of DBS extraction was between 94.1% and 97.2%. No significant linezolid degradation was observed after storing DBS at 50°C for at least one week and at 37°C or ambient temperature for two months as biases were less than 15%.

Variation of blood spot volume between 30 µL to 90 µL had a minor impact on the assay accuracy as the bias ranged from -11.6% to 7.1%. The variation of Hct from 20% to 50% yielded biases within -7.6% to

6.8% and -12.5% to 5.7% for MED and HIGH level. Larger biases of -17.8% to 11.9% were observed at the LOW concentration level (0.25mg/L) (Table 2).

Comparisons of DBS, VDBS and plasma analysis

Significant proportional biases were observed in Passing Bablok regressions in which the slope of regression line between DBS and plasma was 1.28 (95%CI: 1.13-1.44) and VDBS and plasma was 1.46 (95%CI: 1.40-1.54). The intercepts were –0.42 (95%CI: -1.72-0.17) and -0.67 (95%CI: -1.36-0.09), respectively (Fig. 1). In Bland–Altman analysis, the geometric mean concentration ratios of DBS and VDBS versus plasma were 1.20 (95%CI: 1.12-1.27) and 1.36 (95%CI: 1.32-1.40), respectively. The ratio of VDBS/plasma was higher than that of DBS/Plasma (Wilcoxon signed-rank test, n=24, p<0.01). 95% limits of agreement were shown with less than 5% of the values falling out of the ranges (Fig. 2).

Pharmacokinetic and pharmacodynamic evaluation

A median plasma $AUC_{0\text{-}12h}$ of 50.9 (IQR: 50.5-54.9) $mg \cdot h \cdot L^{-1}$ was observed following a dose of 300 mg and 126 (IQR: 121.6-127.6) $mg \cdot h \cdot L^{-1}$ following a dose of 600 mg. Linezolid pharmacokinetic parameters are shown in Table 3. The concentration-time curves of plasma, DBS and VDBS are presented in Fig. 3.

The $AUC_{0\text{-}12h}$ values of DBS and VDBS were calculated using the conversing factors 1.20 and 1.36 for DBS and VDBS respectively. The subsequent result showed a good agreement with plasma. All the values were within the 95% limit of agreement (Fig.4). The individual data for each patient for $AUC_{0\text{-}12h}$ attained from plasma and conversed (V)DBS concentrations and the respective $AUC_{0\text{-}24h}$/MIC values are presented in Table 4. Patients that received a linezolid dose of 300 mg twice daily (n=3) had a median plasma $AUC_{0\text{-}24h}$/MIC ratio of 236 $mg \cdot h \cdot L^{-1}$ (IQR: 219-322 $mg \cdot h \cdot L^{-1}$) and patients that received 600 mg twice daily (n=5) had a median plasma $AUC_{0\text{-}24h}$/MIC ratio of 508 $mg \cdot h \cdot L^{-1}$ (IQR: 486-1398 $mg \cdot h \cdot L^{-1}$).

DISCUSSION

This study showed that DBS analysis is an easy tool to individualize MDR-TB treatment with linezolid. In addition, this report presents a novel, validated method of analysis of linezolid in dried blood spots, with specimens that proved to be very stable over time.

In previous studies on DBS analysis of other drugs, several technical factors were pointed out that have to be considered when interpreting DBS analysis, such as the effect of Hct and blood spot volume (12, 13, 18, 30). For the analysis of linezolid in DBS, the effect of Hct seemed to be of minor concern. In this study, biases fell within accepted ranges for Hcts between 20-50%. These Hcts cover an even broader range than clinical Hcts found in TB patients in literature, i.e. 35.4±6.7% (3), and in this study 37.4±4.4%. Based on these findings, the standardization of Hct at 35% during DBS validation is acceptable. Furthermore, variation of blood spot volume between 30-90μL had little effect as biases were within 15%.

Despite the minor influence of technical factors, i.e. Hct value and blood spot volume, physiological factors are also mentioned in literature to possibly limit the applicability and interpretation of DBS analysis (13). Such a factor might be differences between plasma concentration and whole blood concentration. This study shows that concentration of linezolid is higher in blood than in plasma. This is caused by different binding capacity to plasma proteins and blood cells. Furthermore, concentrations of linezolid were higher in VDBS than in DBS. This might be caused by differences between the capillary and venous blood (13, 25, 30). Nevertheless, the concentration of DBS and VDBS specimens,

both showed good correlation with plasma concentration. To compensate for these differences, we propose conversion factors of 0.83 (1 / 1.20) for DBS and 0.74 (1 / 1.36) for VDBS to calculate corresponding plasma values. After the conversion, good agreement between $AUC_{0\text{-}12h}$ of DBS and plasma was observed.

A meta-analysis showed that a ≤600 mg linezolid daily dose resulted in lower frequency of either adverse event or adverse events necessitating treatment discontinuation than the dose of >600 mg daily (8). Among the published data, the lowest rate of adverse effects was observed with a dose of 300 mg once daily (17). Nevertheless, lowering the dose clearly results in lower exposure to the drug (2, 19). In addition, interpatient variability and possible drug-drug interactions may lead to under- or over-exposure. Therefore, treatment with a fixed dose may be questionable (6, 11, 22, 26). The application of TDM for linezolid can help avoid under- or overexposure which may occur in 30 to 40% of the cases (20).

In this study, all patients had *Mycobacterium tuberculosis* isolates with a MIC ≤0.5mg · L^{-1} for linezolid. With a dose of 600 mg (n=5) twice daily, very high $AUC_{0\text{-}24h}$/MIC ratios were reached(10), so dose reductions could be implemented to prevent time- and dose-dependent toxicity. Furthermore, a high correlation of $AUC_{0\text{-}24h}$/MIC values between conversed DBS and plasma (Spearman's rho=0.976, n=8) was observed. This suggests that TDM using DBS may result in identical interventions compared with conventional plasma sampling. Therefore, adaptive dosing of linezolid to prevent potential toxicity and to assure therapeutic exposure is feasible using DBS.

The high stability of DBS specimens can minimize the logistic burden of conventional sampling in limited-resource areas. With a simple instruction, the DBS samples can be performed easily and sent to equipped facilities for analysis by mail (12, 30). This could enable the TDM in TB-programs worldwide including resource limited settings where MDR/XDR-TB epidemic is a growing problem. TDM using DBS for MDR/XDR-TB should be especially considered in areas where HIV or malaria co-infections are highly prevalent as DBS has been successfully applied to monitor the treatment of such diseases (30).

Since treatment of MDR/XDR-TB is long and complicated by adverse drug reactions, TDM of linezolid with DBS could be used to optimize drug exposure during treatment. In conclusion, this study presents a novel, validated analysis of linezolid in DBS specimens that is suitable for optimization of linezolid treatment of MDR-TB. Advantages include a very simple, low biohazard risk sampling method using a finger prick, easy logistics and very good stability of DBS specimens.

REFERENCES

1. Alffenaar, J. W., J. G. Kosterink, R. van Altena, T. S. van der Werf, D. R. Uges, and J. H. Proost. 2010. Limited sampling strategies for therapeutic drug monitoring of linezolid in patients with multidrug-resistant tuberculosis. Ther. Drug Monit. 32:97-101. doi: 10.1097/FTD.0b013e3181cc6d6f.
2. Alffenaar, J. W., R. van Altena, I. M. Harmelink, P. Filguera, E. Molenaar, A. M. Wessels, D. van Soolingen, J. G. Kosterink, D. R. Uges, and T. S. van der Werf. 2010. Comparison of the pharmacokinetics of two dosage regimens of linezolid in multidrug-resistant and extensively drug-resistant tuberculosis patients. Clin. Pharmacokinet. 49:559-565. doi: 10.2165/11532080-000000000-00000; 10.2165/11532080-000000000-00000.
3. Antony, S. J., V. Harrell, J. D. Christie, H. G. Adams, and R. L. Rumley. 1995. Clinical differences between pulmonary and extrapulmonary tuberculosis: a 5-year retrospective study. J. Natl. Med. Assoc. 87:187-192.
4. Bland, J. M., and D. G. Altman. 1999. Measuring agreement in method comparison studies. Stat. Methods Med. Res. 8:135-160.
5. Bolhuis, M. S., A. D. Pranger, and J. W. Alffenaar. 2012. Linezolid: safety and efficacy monitoring. Eur. Respir. J. 39:.
6. Bolhuis, M. S., R. van Altena, D. R. Uges, T. S. van der Werf, J. G. Kosterink, and J. W. Alffenaar. 2010. Clarithromycin significantly increases linezolid serum concentrations. Antimicrob. Agents Chemother. 54:5418-5419. doi: 10.1128/AAC.00757-10.
7. Conte, J. E.,Jr, J. A. Golden, J. Kipps, and E. Zurlinden. 2002. Intrapulmonary pharmacokinetics of linezolid. Antimicrob. Agents Chemother. 46:1475-1480.
8. Cox, H., and N. Ford. 2012. Linezolid for the treatment of complicated drug-resistant tuberculosis: a systematic review and meta-analysis [Review article. Int. J. Tuberc. Lung Dis. . doi: 10.5588/ijtld.11.0451.
9. Cynamon, M. H., S. P. Klemens, C. A. Sharpe, and S. Chase. 1999. Activities of several novel oxazolidinones against Mycobacterium tuberculosis in a murine model. Antimicrob. Agents Chemother. 43:1189-1191.
10. Dooley, K. E., E. A. Obuku, N. Durakovic, V. Belitsky, C. Mitnick, E. L. Nuermberger, and on behalf of the Efficacy Subgroup, RESIST-TB. 2012. World Health Organization Group V Drugs for the Treatment of Drug-Resistant Tuberculosis: Unclear Efficacy Or Untapped Potential? J. Infect. Dis. . doi: 10.1093/infdis/jis460.
11. Dryden, M. S. 2011. Linezolid pharmacokinetics and pharmacodynamics in clinical treatment. J. Antimicrob. Chemother. 66 Suppl 4:iv7-iv15. doi: 10.1093/jac/dkr072.
12. Edelbroek, P. M., J. van der Heijden, and L. M. Stolk. 2009. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther. Drug Monit. 31:327-336. doi: 10.1097/FTD.0b013e31819e91ce.
13. Emmons, G., and M. Rowland. 2010. Pharmacokinetic considerations as to when to use dried blood spot sampling. Bioanalysis. 2:1791-1796. doi: 10.4155/bio.10.159.
14. Fortun, J., P. Martin-Davila, E. Navas, M. J. Perez-Elias, J. Cobo, M. Tato, E. G. De la Pedrosa, E. Gomez-Mampaso, and S. Moreno. 2005. Linezolid for the treatment of multidrug-resistant tuberculosis. J. Antimicrob. Chemother. 56:180-185. doi: 10.1093/jac/dki148.
15. Gebhart, B. C., B. C. Barker, and B. A. Markewitz. 2007. Decreased serum linezolid levels in a critically ill patient receiving concomitant linezolid and rifampin. Pharmacotherapy. 27:476-479. doi: 10.1592/phco.27.3.476.
16. Harmelink, I. M., J. W. Alffenaar, A. M. Wessels, B. Greijdanus, and D. R. Uges. 2008. A rapid and simple liquid chromatography-tandem mass spectrometry method for the determination of linezolid in human serum. EJHP Science. 14:3-7.
17. Koh, W. J., Y. R. Kang, K. Jeon, O. J. Kwon, J. Lyu, W. S. Kim, and T. S. Shim. 2012. Daily 300 mg dose of linezolid for multidrug-resistant and extensively drug-resistant tuberculosis: updated analysis of 51 patients. J. Antimicrob. Chemother. . doi: 10.1093/jac/dks078.
18. Li, W., and F. L. Tse. 2010. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed. Chromatogr. 24:49-65. doi: 10.1002/bmc.1367.

19. McGee, B., R. Dietze, D. J. Hadad, L. P. Molino, E. L. Maciel, W. H. Boom, M. Palaci, J. L. Johnson, and C. A. Peloquin. 2009. Population pharmacokinetics of linezolid in adults with pulmonary tuberculosis. Antimicrob. Agents Chemother. 53:3981-3984. doi: 10.1128/AAC.01378-08.
20. Pea, F., M. Furlanut, P. Cojutti, F. Cristini, E. Zamparini, L. Franceschi, and P. Viale. 2010. Therapeutic drug monitoring of linezolid: a retrospective monocentric analysis. Antimicrob. Agents Chemother. 54:4605-4610. doi: 10.1128/AAC.00177-10.
21. Prammananan, T., A. Chaiprasert, and M. Leechawengwongs. 2009. In vitro activity of linezolid against multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant (XDR)-TB isolates. Int. J. Antimicrob. Agents. 33:190-191. doi: 10.1016/j.ijantimicag.2008.08.007.
22. Sasaki, T., H. Takane, K. Ogawa, S. Isagawa, T. Hirota, S. Higuchi, T. Horii, K. Otsubo, and I. Ieiri. 2011. Population pharmacokinetic and pharmacodynamic analysis of linezolid and a hematologic side effect, thrombocytopenia, in Japanese patients. Antimicrob. Agents Chemother. 55:1867-1873. doi: 10.1128/AAC.01185-10.
23. Singla, R., J. A. Caminero, A. Jaiswal, N. Singla, S. Gupta, R. K. Bali, and D. Behera. 2012. Linezolid: an effective, safe and cheap drug for patients failing multidrug-resistant tuberculosis treatment in India. Eur. Respir. J. 39:956-962. doi: 10.1183/09031936.00076811.
24. Spooner, N., R. Lad, and M. Barfield. 2009. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. Anal. Chem. 81:1557-1563. doi: 10.1021/ac8022839.
25. Spooner, N., Y. Ramakrishnan, M. Barfield, O. Dewit, and S. Miller. 2010. Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations. Bioanalysis. 2:1515-1522. doi: 10.4155/bio.10.105.
26. Srivastava, S., J. G. Pasipanodya, C. Meek, R. Leff, and T. Gumbo. 2011. Multidrug-resistant tuberculosis not due to noncompliance but to between-patient pharmacokinetic variability. J. Infect. Dis. 204:1951-1959. doi: 10.1093/infdis/jir658.
27. US Department of Health and Human Services Food and Drug Administration. 2001. Guidance for Industry. Bioanalytical Method Validation. 2008:.
28. van Klingeren, B., M. Dessens-Kroon, T. van der Laan, K. Kremer, and D. van Soolingen. 2007. Drug susceptibility testing of Mycobacterium tuberculosis complex by use of a high-throughput, reproducible, absolute concentration method. J. Clin. Microbiol. 45:2662-2668. doi: 10.1128/JCM.00244-07.
29. Vinh, D. C., and E. Rubinstein. 2009. Linezolid: a review of safety and tolerability. J. Infect. 59 Suppl 1:S59-74. doi: 10.1016/S0163-4453(09)60009-8.
30. Vu, D. H., J. W. Alffenaar, P. M. Edelbroek, J. R. Brouwers, and D. R. Uges. 2011. Dried blood spots: a new tool for tuberculosis treatment optimization. Curr. Pharm. Des. 17:2931-2939.
31. Vu, D. H., R. A. Koster, J. W. Alffenaar, J. R. Brouwers, and D. R. Uges. 2011. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci. 879:1063-1070. doi: 10.1016/j.jchromb.2011.03.017.
32. Williams, K. N., C. K. Stover, T. Zhu, R. Tasneen, S. Tyagi, J. H. Grosset, and E. Nuermberger. 2009. Promising antituberculosis activity of the oxazolidinone PNU-100480 relative to that of linezolid in a murine model. Antimicrob. Agents Chemother. 53:1314-1319. doi: 10.1128/AAC.01182-08.
33. World Health Organisation (WHO). 2010. Treatment of tuberculosis: guidelines. World Health Organisation, Geneva.
34. Yew, W. W., C. H. Chau, and K. H. Wen. 2008. Linezolid in the treatment of 'difficult' multidrug-resistant tuberculosis. Int. J. Tuberc. Lung Dis. 12:345-346.

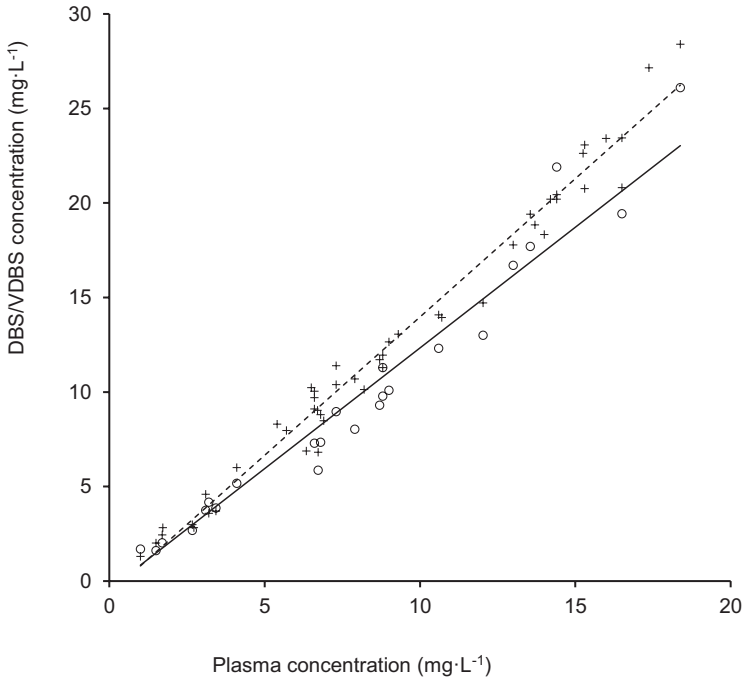

FIG 1

Passing-Bablok regression between measurements in DBS/VDBS samples and plasma: VDBS-plasma regression line (dashed line, cross), slope of 1.46 (95% CI, 1.40 to 1.54) and intercept of -0.67 (95% CI, -1.36 to -0.09); DBS-plasma regression line (solid line, opened circle), slope of 1.28 (95% CI, 1.13 to 1.44) and intercept of -0.42 (95% CI, -1.72 to 0.17).

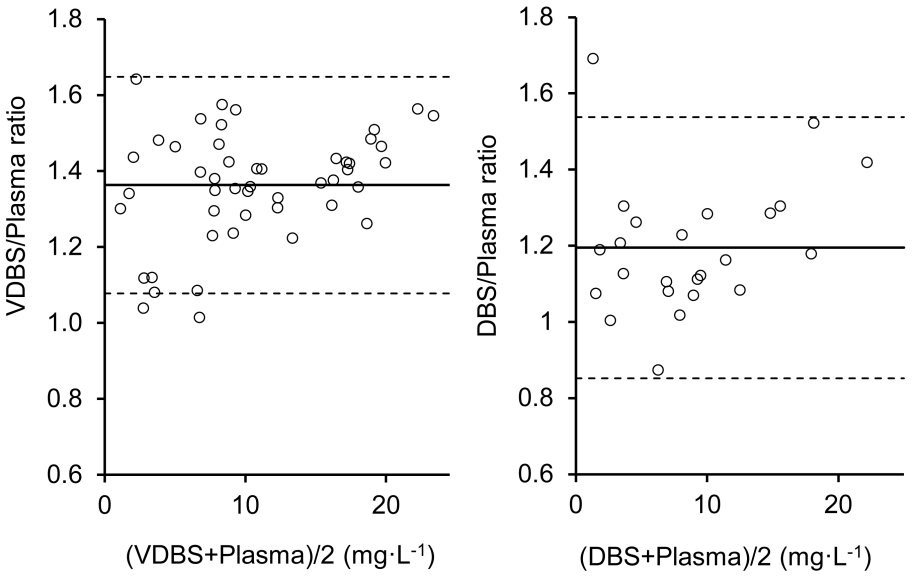

FIG 2

Bland-Altman plot of linezolid concentration ratios in DBS and VDBS samples versus plasma: solid line, mean ratio; dashed line, limit of agreement (mean ratio ± 1.96 × standard deviation of the ratio).

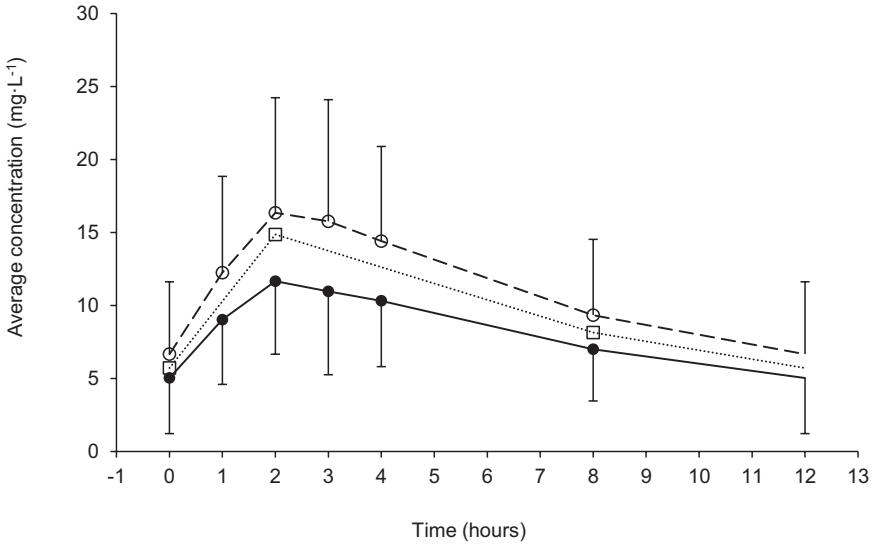

FIG 3

Concentration-time curves of linezolid in plasma (closed circle, solid line), VDBS samples (open circle, dashed line), and DBS samples (opened square, dotted line). Plasma and VDBS data are presented as means and standard deviations. For visual purposes, the DBS data are presented as means without error bars.

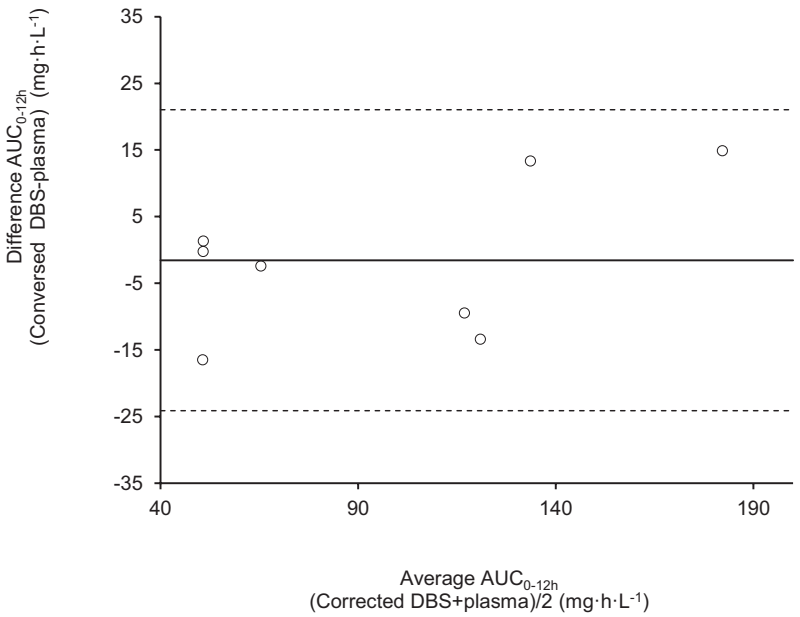

FIG 4

Bland-Altman plot of AUC_{0-12} for linezolid from corrected DBS versus AUC_{0-12} plasma samples. Solid line, mean difference; dashed line, limit of agreement (mean difference ± 1.96 standard deviation of the difference).

Table 1. Baseline demographics of study patients (n=8)

Parameter	Value
Age (y)	29 [24 - 33] [a]
Sex (n)	
Male	2
Female	6
Bodyweight (kg)	60.5 [55.8 - 61.5] [a]
Height (m)	1.69 [1.67 – 1.76] [a]
Body mass index (kg/m^2)	20.1 [19.0 - 21.4] [a]
Ethnicity (no. of patient)	
Caucasian	4
African	3
Asian	1
Co-morbidity (n/N)[d]	
HIV	1/8
Hemoglobin (mmol/liter)	7.0 [6.4 - 8.8] [a, b]
Hematocrit (%)	37.4 [33.0 - 41.4] [a, c]
TB treatment other than linezolid (no. of patient)	
Moxifloxacin	7
Amikacin/Kanamycin	6
Ethambuthol	5
Cotrimoxazole	3
Clofazimine	2
Ertapenem	2
Pyrazinamide	1

[a] Median value (IQR); [b] Reference values for healthy subjects, 7.5 to 9.9 mmol/liter (female) and 8.7 to 10.6 mmol/liter (male); [c] Reference values for healthy subjects, 37.0 to 47.0% (female) and 42.0 to 52.0% (male); [d] n/N, number of TB patients with other infections/total number of TB patients.

Table 2. Summarized results of the validation of DBS analysis

	Validation levels (n=5)[e]			
Validation criteria	LLOQ	LOW	MED	HIGH
Nominal concentrations (mg · L^{-1})	0.05	0.25	15	30
Reproducibility [a]				
Accuracy (% Bias)	4.5	6.3	3.2	-1.3
Within-day precision (% CV)	13.8	4.0	4.1	1.6
Between-day precision (% CV)	10.2	3.5	6.1	7.7
Overall precision (% CV)	17.2	5.3	7.4	7.8
Matrix effect (%)		2.9	8.7	1.9
Recovery (%)		95.5	94.1	97.2
Effect of blood volume (range of % Bias) [b]		-2.9 - 4.1	-11.4 - 7.1	-11.6 - 9
Effect of hematocrit (range of % Bias) [c]		-17.8 - 11.9	-7.6 - 6.8	-12.5 - 5.7
Stability [d]				
1 week at 50°C (% Bias)		6.7		-3.4
2 months at 37°C (%Bias)		-10		-5.9
2 months at ambient temperature (% Bias)		-2.5		-2.0

[a]: data from 3 separated validation days; [b]: comparison with sample of standardized blood spot volume (35µL); [c]: comparison with samples of standardized hematocrit (35%); [d]: present data from the last time of the period only; [e]: number of replicates

Table 3. Steady state pharmacokinetic parameters of linezolid in plasma [a]

Parameter	**300mg (n=3)**	**600mg (n=5)**
$AUC_{0\text{-}12h}$ (mg · h/liter)	50.9 [50.5 – 54.9]	126.9 [121.6 – 127.6]
C_{max} (mg/liter)	8.8 [7.8 – 8.9]	16.5 [14.4 – 16.5]
T_{max} (h)	1.9 [1.9 – 4.8]	1.9 [1.7 – 3.0]
$T_{1/2}$ (h)	4.6 [4.0 – 6.9]	7.5 [7.3 – 7.9]
Cl (liters/h)	4.9 [3.8 – 5.1]	3.1 [3.0 – 3.1]
Vd (liters)	32.6 [29.4 – 34.4]	34.8 [32.9 – 41.6]

[a] Data are presented as medians (IQR); All patients (n = 8) received linezolid twice daily.

Table 4. Pharmacokinetic and pharmacodynamic parameters of linezolid using concentrations in plasma, VDBS, and DBS

Patient	Dose (mg)[a]	MIC	$AUC_{0\text{-}12h}$ (mg · h/liter)			$AUC_{0\text{-}24h}$/MIC		
		(mg/liter)	Plasma	VDBS [b]	DBS [b]	Plasma	VDBS[b]	DBS[b]
1	300	0.5	50.1	46.7	51.5	201	187	206
2	300	0.25	50.9	54.2	50.7	407	433	405
3	600	0.5	121.6	118.1	112.1	486	472	449
4	600	<0.125	127.6	130.9	114.2	>2042	>2094	>1827
5	600	0.5	126.9	132.0	140.2	508	528	561
6	600	0.5	66.6	69.4	64.2	266	278	257
7	300	0.5	58.9	46.1	42.4	236	184	170
8	600	0.25	174.7	183.1	189.6	1398	1465	1517

[a] Twice daily; [b]: relative $AUC_{0\text{-}12h}$ and $AUC_{0\text{-}24h}$/MIC calculated using conversion factors (i.e. 1.20 for DBS and 1.36 for VDBS).

Chapter 8

General discussion and future perspectives

Our research emphasizes new approaches to tuberculosis (TB) control in developing countries where serious and complex TB epidemics may threaten the Millennium Development Goal for TB (2015: halving deaths due to TB compared with baseline of 1990; 2050: eliminating TB as a public health issue) (1). In developing countries, National Tuberculosis control Programs (NTPs) known as public sector play the main role in the TB control mission. Other facilities, including private healthcare, also provide services but seem to be poorly connected with NTP (2,3). Studies show that private healthcare is a potential source of delayed diagnosis of TB, therefore increasing the risk of TB transmission within the community (4,5).

Current therapeutic strategy against TB, updated for the latest scientific developments, has saved many lives. The application of Directly Observed Therapy, Short Course (DOTS) in TB chemotherapy is highly effective, ensuring adherence of the patient to a standardized regime and significantly increasing the cure rate up to 90% (1,6). Nevertheless, the possibly of failed treatment, relapse or development of drug-resistant TB still exists. In addition, the community is being challenged by multidrug-resistant (MDR)/extensively drug-resistant (XDR) tuberculosis and HIV-TB comorbidity. Unfortunately, the numbers of available drugs for MDR/XDR-TB are limited (6). In combination with HIV comorbidity, the high potential of drug-drug interactions further limits the selection for chemotherapy (7,8). The anti-TB drugs left are less effective and more toxic. Currently, about nine compounds are in the pipeline of clinical development and it will take time before they are routinely applied in TB treatment (9,10). While waiting for new effective drugs, optimizing the use of currently available ones is a judicious approach. According to the treatment guideline of WHO, the currently available TB drugs are classified into five groups as presented in the following Table (6).

Table. Antituberculosis drug classification of WHO

Group 1: First-line oral antituberculosis drugs
Isoniazid, rifampicin(#), ethambutol, pyrazinamide, rifabutin
Group 2: Injectable antituberculosis drugs
Kanamycin, amikacin, capreomycin, streptomycin
Group 3: Fluoroquinolones
Levofloxacin, moxifloxacin(#), ofloxacin
Group 4: Oral bacteriostatic second-line antituberculosis drugs
Ethionamide, protionamide, cycloserine, terizidone, p-aminosalicylic acid
Group 5: Antituberculosis drugs with unclear anti-TB efficacy
Clofazimine, linezolid(#), amoxicillin/clavulanate, thioacetazone, clarithromycin(#), imipenem,

(#): Dried blood spot analysis methods are developed

The answer from private pharmacies to TB control

In Vietnam, a high tuberculosis burden developing country, previous studies have identified private pharmacies as the highest potential source causing delay in TB diagnosis and treatment (11,12). Although private pharmacies are not in charge of making confirmed diagnoses of TB cases, they are the healthcare provider that TB patients normally visit to ask for healthcare advice (13-15). The ability to identify a case suspected of TB is therefore important for further advice. If the private pharmacy cannot notice the suspected symptoms of TB, selling non-TB drugs or referring the patient to non-TB healthcare are common-sense decisions (16). Even when patients suspected of TB can be detected,

the decision to refer for diagnosis is still an uncertain one (17). We observed that the ability to detect TB-suspected cases and the willingness of private pharmacies to refer the case to TB healthcare was related to awareness of healthcare providers (HCPs) about the NTP's free treatment policy. A lower-than-expected awareness about free treatment in NTP among HCPs may reflect two issues: (1) the collaboration between NTP and private pharmacies is still insufficient, and (2) the current massive education program for community pharmacists neglects the TB control mission.

The involvement of private pharmacies in dispensing anti-TB drugs is also of great concern. Previous studies show that private pharmacies dispense anti-TB drugs, including self-prescription for TB patients (18) . Because private pharmacies are not commonly updated on the most recent information from the WHO, the treatment outcome is generally poor (19,20). In this study we also found that anti-TB drugs were partly available or unavailable in private pharmacies. Only some anti-TB drugs were intended to be dispensed for TB treatment purpose, and only by prescription. This was confirmed by the simulated patient method, as no self-prescription of anti-TB drugs was generated. This result suggests that NTP and the authority were successful in controlling dispensing of anti-TB drugs in private pharmacies (16). Our study nonetheless addressed potentially improper dispensing of rifampicin as the core first-line drug for susceptible TB, and fluoroquinolones as crucial group for MDR-TB treatment (21). These drugs are commonly available in private pharmacies and are dispensed for the treatment of conditions other than TB, also without prescription. Forty nine (36%) interviewees in private pharmacies claimed that they were willing to dispense fluoroquinolones to suspected TB patient. Using the simulated patient method, eight private pharmacies were willing to dispense fluoroquinolones to a patient suspected of TB. The risk of emerging MDR/XDR TB from a high epidemic area being exposed to such mono-therapies is undeniably possible (22). Further research is warranted to estimate the risk of improper dispensing practices presented in our study.

Dried blood spot as a new tool for TB treatment individualization

Optimal dosage of current anti-TB drugs is still an issue of controversy (23-26). Individual variability in pharmacokinetics is common for anti-TB drugs (27,28). Pharmacokinetic variation is the consequence of a complex combination of factors such as race, genetics, weight, comorbidity, diabetes and HIV. Recent studies show that pharmacokinetic variability is significantly associated with failure of therapy and ADR in patients (29,30). This suggests that individualized dosing for tuberculosis may be more effective than standardized dosing. Furthermore, adverse effects and toxicity commonly occur during the treatment, especially with second-line drugs (31,32). Drug overexposure causing toxicity is difficult to predict if the blood concentration is not measured. Individualized dosing using therapeutic drug monitoring (TDM) can be applied to optimize the treatment (27). Indeed, TDM can help patients with persistent or resistant TB, HIV-TB and diabetes-TB (33). To date, TDM has become practice in the treatment of TB in a limited number of centers with developed logistics and facilities (34-41). However, TDM targets TB patients including MDR/XDR-TB, and HIV-TB can be found more in low- or middle-income countries with limited resources. In these areas, TDM using conventional liquid sampling like serum and plasma faces many logistic obstacles (42). For conventional TDM these may include lack of trained healthcare staff for venous sampling and a cooled chain for sample storage and transportation (42). Recent advances in bioanalytical technology, particularly LC-MS/MS, makes DBS an impressive tool to overcome such obstacles (42,43). We developed methods of analysis using dried blood spot sampling for rifampicin, moxifloxacin, clarithromycin and linezolid, and proved the validity of those methods to determine drug exposure in TB patients. In the classification of the WHO guideline for TB treatment, rifampicin is the core first-line drug for the treatment of susceptible TB, while moxifloxacin

shows to be the most potential fluoroquinolone for the treatment of MDR/XDR-TB. The individual pharmacokinetic variability of rifampicin and moxifloxacin is high and TDM is suggested (28,44-47). Linezolid and clarithromycin are classified as group-5 anti-TB drugs for the treatment of MDR/XDR-TB. Linezolid proved its efficacy on MDR/XDR-TB but its high toxicity suggests individualized treatment may be beneficial (31).

The attractive characteristic of DBS sampling is that the specimens are stable at ambient or high temperature. Our research showed that moxifloxacin, rifampicin, clarithromycin and linezolid are stable at room temperature for up to one or two months. Consequently, equipment to ensure a low temperature for sample storage and transportation is not necessary. At a temperature of 37°C this mimics the conditions in tropical countries or those of 50°C that may occur during transportation; rifampicin, linezolid and clarithromycin showed acceptable stability. These impressive data support that TDM using DBS sampling requires minimal logistics and that individualized dosing for TB outpatients in low- and middle-income areas is feasible (48-50).

We reviewed recent papers to gather experience of previous studies to facilitate the development of our methods (42). Our results provided deeper understanding about previous opinions on analytical and sampling technique of dried blood spots. We emphasized that hematocrit and blood spot volume impacted on analytical results to some extent. In general, analytical concentrations of all investigated drugs showed upward trend biases as blood spot volume increases. However, this effect was trivial and could be omitted. Therefore, DBS specimens can be collected regardless of precise volumetric devices like pipettes, and this is much convenient for sampling in resource-limited areas. Extreme hematocrit values may introduce significant biases (more than 15%) to the analytical results of drugs. The viscosity of the blood appears to be the main influencing factor because correcting for spot size can neutralize such bias (16,42,49,50). Hematocrit values of patients necessary for calculating the correction are normally available in medical records. It is noticeable that the extreme values of hematocrit at 20% or 50% presented in analytical method development are not common among TB patients. The DBS analytical results can therefore be interpreted without correction, as bias remains below 15%. With extremely low or high hematocrit, clinical pharmacists may consider correction if they find it necessary.

During development of analytical methods using LC-MS/MS matrix effect and carry-over may be hurdles to overcome. We presented a logical approach to troubleshoot the carry-over when analyzing rifampicin in plasma (51). Without carry-over this method is a good starting point for developing a DBS analytical method. Endogenous components from the DBS had a significant influence on the results. As high sample throughput should be realized for methods of analysis to be performed in daily practice, solid phase or liquid-liquid extraction should be avoided. Interaction between rifampicin and endogenous components in DBS was explored, and it appeared that adding complexing agents could suppress this effect. The validated method with simple extraction and precipitation is very quick, providing a high sample throughput (49).

The clinical validation in TB patients made the developed analytical methods applicable to clinical pharmacokinetics studies and to individualized patient care. To be able to compare DBS analytical results with the available pharmacokinetic data, which is mainly obtained based on plasma/serum sampling, clinical validation in TB patients had to be performed. Biases between the concentrations in DBS and plasma, which may have resulted from numerous physiological factors, are common (42). In our research, biases observed were negative or positive depending on the drugs (48-50). These biases

can be minimized by correcting with conversing factors attained in clinical validation. Corrected DBS concentrations after conversion can predict the plasma level. Our study demonstrates that using dried blood spot, PK/PD parameters can be accurately estimated. A high correlation of linezolid $AUC_{0\text{-}24h}$/MIC values between conversed DBS and plasma (Spearman's rho=0.976, n=8) was observed. With the standard dose of 600 mg (n=5) twice daily, very high $AUC_{0\text{-}24h}$/MIC ratios were reached (52). Dose reductions should therefore be implemented to prevent time- and dose-dependent toxicity. This suggests that TDM using DBS may result in identical interventions compared with conventional plasma sampling. Adaptive dosing of linezolid to prevent potential toxicity and to assure therapeutic exposure is feasible using DBS (48). Because therapeutic exposure can be determined by using DBS sampling and analysis, patients with malabsorption or poor adherence can be detected (49). The drug resistance that emerges from monotherapy of concomitant TB drugs can be minimized.

A previous study presented that DBS sampling is easy to perform and with minimal invasion to patients (53). Although no such evaluation was carried out during clinical validation of our study, we did not encounter difficulties with DBS sampling and all samples were collected as planned. In addition, analytical methods were developed with a run time of three to four minutes per sample, ensuring the laboratory could handle a large number of measurements (48-50). Easy sampling, simple sample preparation and throughput analytical method can further facilitate the application of DBS in an actual clinical setting.

Future perspectives

Our work contributes to new scientific and programmatic views for TB control strategies, especially in developing countries like Vietnam. The results of our research strengthen our belief that programmatic intervention on the knowledge of HCPs in private pharmacies can improve TB referrals and shorten delays. The involvement of private pharmacies in the public-private mix program can reduce delays, save healthcare costs and increase case detection. As education is one component of Good Pharmacy Practice (GPP) for pharmacists in private pharmacies, our finding suggests that the authority who certifies GPP may consider incorporating knowledge of TB control into this aspect.

By using DBS sampling the logistic obstacles of conventional TDM could be omitted. We propose a programmatic paradigm of TDM for TB treatment in which the DOTS practice, connected with a central, well-equipped laboratory and clinic, play an important role (Figure).

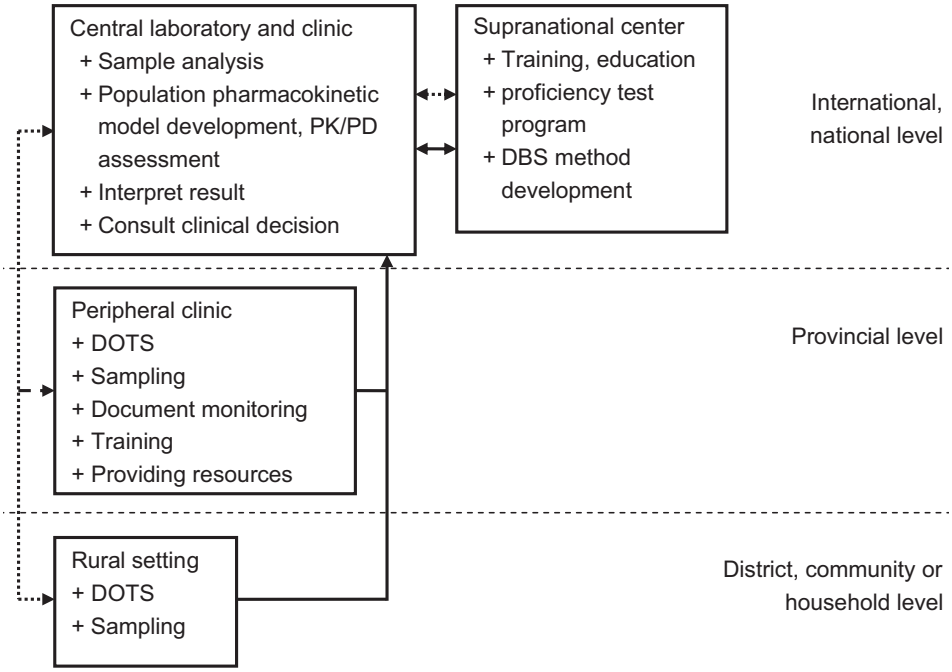

Figure.

Programmatic paradigm for implementation of TDM on TB patients using dried blood spot sampling; solid arrows: sample transport; dashed arrows: information communication.

TDM requires multidisciplinary collaboration therefore application should preferably be limited to a targeted group of patients only. In combination with limited sampling strategies, DBS can be used for further individualization of TB treatment (54). Clinical pharmacists should be involved in this procedure to give advice to physicians regarding optimal dosage. Skeptics may raise questions about the high cost of TDM services yet they should consider the extreme cost for treatment of MDR/XDR-TB that may be prevented by TDM (55). A study on costs and benefits of DBS may provide the answer to this question.

With the advance of DBS sampling, longitudinal data of pharmacokinetic measurements can be feasible. Longitudinal data with repeated measurement during treatment is necessary to evaluate the within-patient variability in pharmacokinetics parameters which is still not well-investigated (27).

Within the limits of this thesis we developed analytical methods for four representative anti-TB drugs, and proved the feasibility of DBS sampling for treatment individualization. Further analytical methods for other TB drugs are required to convey this tool to the clinical setting. With the highly effective drugs in group 1, group 2 and group 3 of the WHO classification, treatment individualization to ensure sufficient exposure can be performed in special subgroups of patients who are at risk of failure or run the risk of progressing to MDR/XDR-TB. With the drugs in group 4 or group 5, which contribute to MDR/XDR-TB treatment, high toxicity may be problem of concern and TDM is a reasonable option.

Developing DBS analytical methods for agents in the pipeline of clinical development may accelerate the application of this tool in the future. Cautious use of such new agents is required to preserve their pharmacological efficacy.

REFERENCES

(1) World Health Organization. Global tuberculosis report 2012. Genever, Switzeland: WHO: World Health Organization; 2012.

(2) Bell CA, Duncan G, Saini B. Knowledge, attitudes and practices of private sector providers of tuberculosis care: a scoping review. Int J Tuberc Lung Dis 2011 Aug;15(8):1005-1017.

(3) Rajeswari R, Balasubramanian R, Bose MS, Sekar L, Rahman F. Private pharmacies in tuberculosis control--a neglected link. Int J Tuberc Lung Dis 2002 Feb;6(2):171-173.

(4) Storla DG, Yimer S, Bjune GA. A systematic review of delay in the diagnosis and treatment of tuberculosis. BMC Public Health 2008 Jan 14;8:15.

(5) Dye C, Williams BG. The population dynamics and control of tuberculosis. Science 2010 May 14;328(5980):856-861.

(6) WHO global Tuberculsois.Stop TB Dept. Treatment of tuberculosis guidelines [WHO/HTM/TB/2009.420]. : World Health Organization; 2010.

(7) Chideya S, Winston CA, Peloquin CA, Bradford WZ, Hopewell PC, Wells CD, et al. Isoniazid, rifampin, ethambutol, and pyrazinamide pharmacokinetics and treatment outcomes among a predominantly HIV-infected cohort of adults with tuberculosis from Botswana. Clin Infect Dis 2009 Jun 15;48(12):1685-1694.

(8) Breen RAM, Swaden L, Ballinger J, Lipman MCI. Tuberculosis and HIV co-infection - A practical therapeutic approach. Drugs 2006;66(18):2299-2308.

(9) Lienhardt C, Vernon A, Raviglione MC. New drugs and new regimens for the treatment of tuberculosis: review of the drug development pipeline and implications for national programmes. Curr Opin Pulm Med 2010 May;16(3):186-193.

(10) Koul A, Arnoult E, Lounis N, Guillemont J, Andries K. The challenge of new drug discovery for tuberculosis. Nature 2011 Jan 27;469(7331):483-490.

(11) Huong NT, Vree M, Duong BD, Khanh VT, Loan VT, Co NV, et al. Delays in the diagnosis and treatment of tuberculosis patients in Vietnam: a cross-sectional study. BMC Public Health 2007;7(1471-2458):110.

(12) Lonnroth K, Thuong LM, Linh PD, Diwan VK. Delay and discontinuity--a survey of TB patients' search of a diagnosis in a diversified health care system. Int J Tuberc Lung Dis 1999 Nov;3(11):992-1000.

(13) Hoa NB, Tiemersma EW, Sy DN, Nhung NV, Vree M, Borgdorff MW, et al. Health-seeking behaviour among adults with prolonged cough in Vietnam. Trop Med Int Health 2011 Oct;16(10):1260-1267.

(14) Smith F. The quality of private pharmacy services in low and middle-income countries: a systematic review. Pharm World Sci 2009 Jun;31(3):351-361.

(15) Smith F. Private local pharmacies in low- and middle-income countries: a review of interventions to enhance their role in public health. Trop Med Int Health 2009 Mar;14(3):362-372.

(16) Vu DH, van Rein N, Cobelens FG, Nguyen TT, Le VH, Brouwers JR. Suspected tuberculosis case detection and referral in private pharmacies in Viet Nam. Int J Tuberc Lung Dis 2012 Dec;16(12):1625-1629.

(17) Lonnroth K, Karlsson M, Lan NT, Buu TN, Dieu TT. Referring TB suspects from private pharmacies to the National Tuberculosis Programme: experiences from two districts in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 2003 Dec;7(12):1147-1153.

(18) Lonnroth K, Lambregts K, Nhien DT, Quy HT, Diwan VK. Private pharmacies and tuberculosis control: a survey of case detection skills and reported anti-tuberculosis drug dispensing in private pharmacies in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 2000 11;4(1027-3719; 11):1052-1059.

(19) Hurtig AK, Pande SB, Baral SC, Porter JD, Bam DS. Anti-tuberculosis treatment in private pharmacies, Kathmandu Valley, Nepal. Int J Tuberc Lung Dis 2000 Aug;4(8):730-736.
(20) Kamat VR, Nichter M. Pharmacies, self-medication and pharmaceutical marketing in Bombay, India. Soc Sci Med 1998 Sep;47(6):779-794.
(21) Vu DH, van Rein N, Cobelens FG, Nguyen TT, Le VH, Brouwers JR. Dispensing of anti-tuberculosis drugs in private pharmacies in Viet Nam. In preparation.
(22) Wang JY, Hsueh PR, Jan IS, Lee LN, Liaw YS, Yang PC, et al. Empirical treatment with a fluoroquinolone delays the treatment for tuberculosis and is associated with a poor prognosis in endemic areas. Thorax 2006 Oct;61(10):903-908.
(23) Gumbo T, Louie A, Deziel MR, Parsons LM, Salfinger M, Drusano GL. Selection of a moxifloxacin dose that suppresses drug resistance in Mycobacterium tuberculosis, by use of an in vitro pharmacodynamic infection model and mathematical modeling. J Infect Dis 2004 11/01;190(0022-1899; 0022-1899; 9):1642-1651.
(24) Steingart KR, Jotblad S, Robsky K, Deck D, Hopewell PC, Huang D, et al. Higher-dose rifampin for the treatment of pulmonary tuberculosis: a systematic review. Int J Tuberc Lung Dis 2011 Mar;15(3):305-316.
(25) Koh WJ, Kang YR, Jeon K, Kwon OJ, Lyu J, Kim WS, et al. Daily 300 mg dose of linezolid for multidrug-resistant and extensively drug-resistant tuberculosis: updated analysis of 51 patients. J Antimicrob Chemother 2012 Mar 8.
(26) Gumbo T. New susceptibility breakpoints for first-line antituberculosis drugs based on antimicrobial pharmacokinetic/pharmacodynamic science and population pharmacokinetic variability. Antimicrob Agents Chemother 2010 Apr;54(4):1484-1491.
(27) McIlleron H, Wash P, Burger A, Norman J, Folb PI, Smith P. Determinants of rifampin, isoniazid, pyrazinamide, and ethambutol pharmacokinetics in a cohort of tuberculosis patients. Antimicrob Agents Chemother 2006 Apr;50(4):1170-1177.
(28) Pranger AD, van Altena R, Aarnoutse RE, van Soolingen D, Uges DR, Kosterink JG, et al. Evaluation of moxifloxacin for the treatment of tuberculosis: 3 years of experience. Eur Respir J 2011 Oct;38(4):888-894.
(29) Srivastava S, Pasipanodya JG, Meek C, Leff R, Gumbo T. Multidrug-resistant tuberculosis not due to noncompliance but to between-patient pharmacokinetic variability. J Infect Dis 2011 Dec 15;204(12):1951-1959.
(30) Pasipanodya JG, Srivastava S, Gumbo T. Meta-analysis of clinical studies supports the pharmacokinetic variability hypothesis for acquired drug resistance and failure of antituberculosis therapy. Clin Infect Dis 2012 Jul;55(2):169-177.
(31) Lee M, Lee J, Carroll MW, Choi H, Min S, Song T, et al. Linezolid for treatment of chronic extensively drug-resistant tuberculosis. N Engl J Med 2012 Oct 18;367(16):1508-1518.
(32) Bloss E, Kuksa L, Holtz TH, Riekstina V, Skripconoka V, Kammerer S, et al. Adverse events related to multidrug-resistant tuberculosis treatment, Latvia, 2000-2004. Int J Tuberc Lung Dis 2010 MAR;14(3):275-281.
(33) Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis. Drugs 2002;62(0012-6667; 15):2169-2183.
(34) Magis-Escurra C, van den Boogaard J, Ijdema D, Boeree M, Aarnoutse R. Therapeutic drug monitoring in the treatment of tuberculosis patients. Pulm Pharmacol Ther 2012 Feb;25(1):83-86.
(35) Babalik A, Babalik A, Mannix S, Francis D, Menzies D. Therapeutic drug monitoring in the treatment of active tuberculosis. Can Respir J 2011 Jul-Aug;18(4):225-229.
(36) Alffenaar JW, Kosterink JG, van Altena R, van der Werf TS, Uges DR, Proost JH. Limited sampling strategies for therapeutic drug monitoring of linezolid in patients with multidrug-resistant tuberculosis. Ther Drug Monit 2010 Feb;32(1):97-101.
(37) Heysell SK, Moore JL, Keller SJ, Houpt ER. Therapeutic Drug Monitoring for Slow Response to Tuberculosis Treatment in a State Control Program, Virginia, USA. Emerging Infectious Diseases 2010 OCT;16(10):1546-1553.

(38) Holland DP, Hamilton CD, Weintrob AC, Engemann JJ, Fortenberry ER, Peloquin CA, et al. Therapeutic Drug Monitoring of Antimycobacterial Drugs in Patients with Both Tuberculosis and Advanced Human Immunodeficiency Virus Infection. Pharmacotherapy 2009 MAY;29(5):503-510.

(39) Unsalan S, Sancar M, Bekce B, Clark PM, Karagoz T, Izzettin FV, et al. Therapeutic monitoring of isoniazid, pyrazinamide and rifampicin in tuberculosis patients using LC. Chromatographia 2005 JUN;61(11-12):595-598.

(40) Li J, Burzynski JN, Lee YA, Berg D, Driver CR, Ridzon R, et al. Use of therapeutic drug monitoring for multidrug-resistant tuberculosis patients. Chest 2004 12;126(0012-3692; 0012-3692; 6):1770-1776.

(41) Yew WW. Therapeutic drug monitoring in antituberculosis chemotherapy: clinical perspectives. Clin Chim Acta 2001 11;313(0009-8981; 1-2):31-36.

(42) Vu DH, Alffenaar JW, Edelbroek PM, Brouwers JR, Uges DR. Dried blood spots: a new tool for tuberculosis treatment optimization. Curr Pharm Des 2011;17(27):2931-2939.

(43) Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. Ther Drug Monit 2009 06;31(3):327-336.

(44) Manika K, Chatzika K, Zarogoulidis K, Kioumis I. Moxifloxacin in multidrug-resistant tuberculosis: is there any indication for therapeutic drug monitoring? Eur Respir J 2012 Oct;40(4):1051-1053.

(45) van Ingen J, Aarnoutse R, de Vries G, Boeree MJ, van Soolingen D. Low-level rifampicin-resistant Mycobacterium tuberculosis strains raise a new therapeutic challenge. Int J Tuberc Lung Dis 2011 Jul;15(7):990-992.

(46) Nijland HM, Ruslami R, Suroto AJ, Burger DM, Alisjahbana B, van Crevel R, et al. Rifampicin reduces plasma concentrations of moxifloxacin in patients with tuberculosis. Clin Infect Dis 2007 10/15;45(8):1001-1007.

(47) van Crevel R, Alisjahbana B, de Lange WCM, Borst F, Danusantoso H, van der Meer JWM, et al. Low plasma concentrations of rifampicin in tuberculosis patients in Indonesia. Int J Tuberc Lung Dis 2002 JUN;6(6):497-502.

(48) Vu DH, Bolhuis MS, Koster RA, Greijdanus B, de Lange WC, V Altena R, et al. Dried blood spot analysis for therapeutic drug monitoring of linezolid in MDR-TB patients. Antimicrob Agents Chemother 2012 Aug 27.

(49) Vu DH, Koster RA, Bolhuis MS, Altena RV, Nguyen DH, Brouwers JRBJ, et al. Simultaneously determination of rifampicin, clarithromycin and their metabolites in dried blood spots using LC-MS/MS. Submitted.

(50) Vu DH, Koster RA, Alffenaar JW, Brouwers JR, Uges DR. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. J Chromatogr B Analyt Technol Biomed Life Sci 2011 May 1;879(15-16):1063-1070.

(51) Vu DH, Koster RA, Wessels AM, Greijdanus B, Alffenaar JWC, Uges DRA. Troubleshooting carry-over of LC-MS/MS method for rifampicin, clarithromycin and metabolites in human plasma. Submitted.

(52) Dooley KE, Obuku EA, Durakovic N, Belitsky V, Mitnick C, Nuermberger EL, et al. World Health Organization Group 5 Drugs for the Treatment of Drug-Resistant Tuberculosis: Unclear Efficacy or Untapped Potential? J Infect Dis 2012 Aug 30.

(53) Boer T, Wieling J, Meulman E, Reuvers M, Renkema G, Daas I, et al. Application of dried blood spot sampling combined with LC-MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. Biomed Chromatogr 2011 Feb 1.

(54) Alffenaar JW. Dried blood spot analysis combined with limited sampling models can advance therapeutic drug monitoring of tuberculosis drugs. J Infect Dis 2012 Jun;205(11):1765-6; author reply 1766.

(55) Suarez PG, Floyd K, Portocarrero J, Alarcon E, Rapiti E, Ramos G, et al. Feasibility and cost-effectiveness of standardised second-line drug treatment for chronic tuberculosis patients: a national cohort study in Peru. Lancet 2002 Jun 8;359(9322):1980-1989.

Chapter 9

Summary

SUMMARY

The Millennium Development Goal for tuberculosis (TB) of halving deaths due to TB in 2015 compared with the baseline of 1990 and eliminating TB as a public health problem by 2050 is challenged by problems such as the emerged drug resistance and the synergy with HIV infection. This thesis emphasized the clinical pharmacology aspects related to TB control in the developing world, where TB epidemics are serious and complex.

In **Part 1** of this thesis we performed a field study using simulated patient methods and interviews in sequence, to explore the TB control activities of healthcare workers (HCPs) in private pharmacies in Hanoi, Vietnam. In **Chapter 2** we addressed the fact that nearly half of HCPs in private pharmacies do not refer suspected tuberculosis patients, possibly contributing to the delay in diagnosis and treatment. This delay can contribute to worsening the epidemic in such urban areas because patients with active TB can increase the transmission of this disease to the community. Only 11 (9%) HCPs referred a simulated tuberculosis patient to a tuberculosis care facility and were considered to cause no delay. In addition, we detected factors that predict tuberculosis case detection skills and referral decisions in private pharmacies. Using penalized logistic regression, we explored that the awareness of free treatment in the National Tuberculosis Program (NTP) predicted a higher rate of direct referral to TB facilities and a better ability to detect TB from a paper, fictitious case. Those Pharmacies certified with Good Pharmacy Practice (GPP) were less likely to refer simulated patients to TB facilities than non-GPP pharmacies.

Chapter 3 described the availability of first-line and second-line anti-TB drugs in private pharmacies. In general, anti-TB drugs were not frequently presented in private pharmacies. However, private pharmacies nearby the two TB hospitals of Hanoi were more likely to store anti-TB drugs. This subgroup may contribute more to TB control activity in a public-private mix strategy than referral of TB suspects only. Improper dispensing of rifampicin and fluoroquinolones was also addressed. Approximately 31% of the pharmacies dispensed rifampicin for covering an open wound, while about 36% of pharmacies were willing to sell fluoroquinolones to suspected patients. Actually, simulated patients obtained fluoroquinolones from eight private pharmacies for highly suspected TB symptoms. Because fluoroquinolones are back-bone drugs for multidrug-resistant TB (MDR-TB), the dispensing of these drugs should deserve more attention.

In **Part 2** we emphasized dried blood spot (DBS) sampling and analysis of anti-TB drugs, which can applied to treatment optimization. The patient variation in pharmacokinetics of anti-TB drugs is reported to be common. The consequence is that many patients treated with a standardized regimen can have subtherapeutic drugs levels, while others may suffer toxicity because of overexposure. Recent clinical model and meta-analysis reveal that pharmacokinetic variability is responsible for treatment failure and the emerged drug-resistance. The development of a tool for easily implementing therapeutic drug monitoring in the developing world, where TB epidemic is an increasing problem, is required.

In **chapter 4** a review on recent DBS analysis methods was performed to identify the pros and cons of DBS sampling and analytical methods. The opportunity to apply DBS to TB control in an actual resource-limited setting was also discussed. Technical and clinical aspects of DBS analytical method development were summarized in order to support the future appropriate establishment and application of this tool in a clinical environment.

In **chapter 5**, a simple and fast analytical method was developed to determine moxifloxacin in DBS specimens using LC-MS/MS. Moxifloxacin is potential fluoroquinolones against MDR-TB, and has been evaluated in a phase-3 study. The developed method has shown to be linear, selective, accurate and precise. Critical factors including hematocrit, blood spot volume that may impact the accuracy and precision of the method were also evaluated. Blood spot volume appeared to have a minor impact on the accuracy and the precision of the method; therefore use of a volumetric device is not necessary. Hematocrit showed a proportional correlation with the analytical bias, and for patients with extreme hematocrit value correction could be considered. The impact of hematocrit on the analytical bias partly originated from the spreading of blood on paper due to its viscosity. In the clinical validation, DBS concentration showed a high linear correlation with plasma concentration (r2=0.966). The slope is 1.49 (95% confident interval 1.32-1.77), suggesting that moxifloxacin concentrated more in blood cells than in plasma. Moxifloxacin showed a high stability in DBS specimens in which no significant degradation occurred at ambient temperature after at least one month.

In **Chapter 6a** and **Chapter 6b** a throughput method was developed for simultaneous determination of rifampicin, clarithromycin and their active metabolites, 25-desacetyl rifampicin and 14 hydroxy clarithromycin in DBS specimens. Rifampicin is a back-bone first-line anti-TB drug with a high sterilizing activity in vivo. The high variability in patient pharmacokinetics and the controversy about the optimal dose of this drug suggest that TDM is beneficial. First, we describe a stepwise approach to trouble-shoot the carry-over observed in previously developed methods. By replacing the polar end-capped C18 phase column, the carry-over was eliminated (**Chapter 6a**). Second, we applied this finding to develop an analytical method for DBS specimens. By incorporating ethylenediaminetetraacetic acid (EDTA) and deferoxamin in the extraction, the interaction between rifampicin and the endogenous component of DBS causing a bad "matrix effect" was precluded and the overall process efficiency was improved. The clinical validation showed a good correlation between DBS and plasma concentrations of rifampicin, clarithromycin and 14 hydroxy clarithromycin, suggesting that DBS analytical results can be used to predict plasma exposure. Also, the concentrations detected under lower limit of quantification were identical when using DBS and plasma specimens. DBS samples can therefore be collected to detect nonadherence of patients. To enable reliable results when applying DBS sampling in resource-limited settings, a comprehensive stability test was implemented. In contrast with low stability in liquid samples, rifampicin showed to be highly stable in DBS specimens (ambient temperature for 2 months, 37°C for 10 days and 50°C for 3 days). The sample could then be stored and transported without the need for equipment to ensure low temperature conditions (**Chapter 6b**).

In **Chapter 7**, the opportunity for applying DBS sampling and analysis to the TDM was described more in detail with linezolid. A developed and validated DBS analytical method proved reproducible and robust. Clinical validation was implemented by comparing DBS with plasma results using Passing-Bablok regression and Bland-Altman analysis. The ratio of the concentration of linezolid in DBS samples to that in plasma was 1.2 (95% confidence interval 1.12 to 1.27). Linezolid exposure calculated from concentrated DBS samples and plasma showed good agreement. A high correlation of AUC_{0-24}/MIC values between DBS and plasma (Spearman's rho = 0.976, n = 8) was observed. This suggests that TDM using DBS may result in interventions identical to those with conventional plasma sampling. Furthermore, linezolid showed a high stability in DBS specimens at 37°C for 2 months and even at the high temperature of 50°C for one week. Hence adaptive dosing of linezolid to prevent potential toxicity and to ensure therapeutic exposure is feasible using DBS sampling even in limited-resources areas.

In **Chapter 8** we discussed the impact of our results on TB control in the developing world. The position of private pharmacies in the TB control strategy of the National Tuberculosis Program of Vietnam was addressed and revised. The requirement of individualization of TB chemotherapy in the developing world using DBS sampling was discussed. We proposed a programmatic paradigm for TDM implementation supporting clinical decisions in the treatment of TB in limited-resource areas using DBS sampling and analysis.

TÓM TẮT

Mục tiêu thiên niên kỷ của tổ chức y tế thế giới đối với bệnh lao là tới năm 2015 sẽ giảm một nửa số ca tử vong do lao so với năm 1990 và tới năm 2050 sẽ loại bỏ bệnh lao khỏi danh sách các bệnh cộng đồng. Tuy nhiên, mục tiêu này hiện đang bị thách thức do sự phát triển của tình trạng lao kháng thuốc và đồng nhiễm lao với HIV. Luận án này tập trung vào một số vấn đề dược lý lâm sàng liên quan đến việc kiểm soát bệnh lao ở các nước đang phát triển, nơi thường có tình hình dịch tễ lao nghiêm trọng và phức tạp.

Trong **phần 1**, chúng tôi thực hiện một nghiên cứu thực địa có sử dụng phương pháp đóng vai bệnh nhân đi kèm với phương pháp phỏng vấn nhằm tìm hiểu về hoạt động của các nhân viên y tế tại nhà thuốc tư nhân trong công tác phòng chống lao tại Hà Nội. Trong **chương 2**, chúng tôi chỉ ra rằng có tới gần một nửa nhân viên y tế tại nhà thuốc tư nhân không giới thiệu bệnh nhân nghi ngờ lao đi khám làm chậm trễ việc chẩn đoán xác định khởi đầu điều trị. Việc chậm trễ này có thể làm xấu thêm tình hình dịch tễ tại một khu vực nội thành đông dân cư như Hà Nội bởi vì bệnh nhân trong giai đoạn lao tiến triển có thể gây tăng lây nhiễm ra cộng đồng. Chỉ có 11 (9%) nhân viên y tế giới thiệu người đóng vai bệnh nhân đến các cơ sở khám và điều trị lao và do đó có thể coi là không gây chậm trễ trong việc chẩn đoán. Hơn nữa, chúng tôi cũng chỉ ra các yếu tố có liên quan đến kĩ năng phát hiện bệnh nhân lao và quyết định giới thiệu đi khám của nhân viên tại nhà thuốc. Bằng phân tích hồi quy penalized logistic, chúng tôi thấy rằng việc các nhân viên y tế ý thức về chính sách khám và điều trị miễn phí tại Chương trình chống lao quốc gia sẽ có khả năng phát hiện ca lao từ ca giả định tốt hơn cũng như sẽ giới thiệu bệnh nhân nghi ngờ lao đi khám lao nhiều hơn. Các nhà thuốc có chứng chỉ thực hành nhà thuốc tốt dường như ít giới thiệu trực tiếp đến các cơ sở khám lao hơn.

Trong **chương 3**, chúng tôi mô tả về tính sẵn có và việc cung cấp thuốc chống lao tuyến một và tuyến hai tại nhà thuốc tư nhân. Nhìn chung, các thuốc chống lao không xuất hiện nhiều ở các nhà thuốc tư nhân. Tuy nhiên, các nhà thuốc tư nhân gần hai bệnh viện lao tại Hà Nội có vẻ như lưu trữ thuốc điều trị lao nhiều hơn. Do đó, nhóm nhà thuốc này có thể tham gia nhiều hơn vào các hoạt động trong chiến lược phối hợp công tư về phòng chống lao chứ không chỉ thực hiện việc giới thiệu bệnh nhân nghi ngờ lao. Việc bán rifampicin và các flouroquinolone một cách không hợp lý cũng được chỉ ra trong nghiên cứu này. Có khoảng 31% số nhà thuốc bán rifampicin để rắc vào vết thương trong khi 36% nhà thuốc sẵn sàng bán các thuốc thuộc nhóm fluoroquinolone cho bệnh nhân nghi ngờ lao giả định. Trên thực tế, người đóng vai bệnh nhân lao đã mua được fluoroquinolone ở tám nhà thuốc nhằm điều trị các triệu chứng rõ rệt của bệnh lao. Do fluoroquinolone là nhóm thuốc xương sống trong điều trị lao kháng thuốc, việc bán và phân phối các thuốc này cần phải được lưu tâm hơn nữa.

Trong **phần 2**, chúng tôi tập trung vào phương pháp lấy mẫu và phân tích vệt máu khô (Dried Blood Spot, DBS) áp dụng cho các thuốc chống lao để từ đó có thể ứng dụng trong việc tối ưu hóa điều trị. Các nghiên cứu trước đây cho thấy sự biến thiên dược động học của các thuốc chống lao giữa các bệnh nhân là phổ biến. Kết quả là có rất nhiều bệnh nhân đươc điều tri với phác đồ chuẩn sẽ có nồng độ

thuốc dưới ngưỡng điều trị trong khi có các bệnh nhân khác có thể chịu độc tính do nồng độ quá cao. Phân tích meta và mô hình dựa trên số liệu lâm sàng trong các nghiên cứu gần đây cho thấy sự biến thiên dược động học có liên quan đến việc thất bại trong điều trị cũng như làm phát sinh kháng thuốc. Do đó, cần thiết phải xây dựng một công cụ phục vụ cho thực hành giám sát nồng độ thuốc trong điều trị tại các nước đang phát triển, nơi tình hình dịch tễ lao là một vấn đề ngày càng lớn.

Trong **chương 4**, một bài tổng quan đã được viết nhằm xác định các ưu khuyết điểm của phương pháp lấy mẫu và phân tích DBS. Chương này cũng thảo luận về cơ hội áp dụng phương pháp vệt máu khô trong điều trị lao tại các khu vực có nguồn lực hạn chế. Các vấn đề về kĩ thuật cũng như lâm sàng cần thiết cho việc phát triển phương pháp DBS cũng được tổng kết nhằm hỗ trợ xây dựng cũng như ứng dụng phương pháp này vào điều kiện thực tế lâm sàng.

Chương 5 mô tả việc xây dựng phương pháp định lượng moxifloxacin trong mẫu DBS. Moxifloxacin là một kháng sinh trong nhóm fluoroquinolone có hiệu lực với lao kháng thuốc và hiện đang được thử nghiệm lâm sàng pha 3. Phương pháp được xây dựng đảm bảo tính tuyến tính, tính chọn lọc, độ đúng và độ chính xác. Các yếu tố quan trọng có thể ảnh hưởng đến độ đúng độ chính xác của phương pháp như hematocrit, thể tích mẫu máu cũng được nghiên cứu. Thể tích mẫu máu không ảnh hưởng nhiều tới độ đúng và độ chính xác của phương pháp nên không cần phải sử dụng dụng cụ đo thể tích chính xác trong quá trình lấy mẫu. Hemtocrit có tương quan thuận với sai số hệ thống của phương pháp phân tích và do đó với các bệnh nhân có giá trị hematocrit quá cao hoặc quá thấp thì việc hiệu chỉnh có thể được cân nhắc. Khả năng dàn trải khác nhau của máu trên mặt giấy do độ nhớt khác nhau có thể giải thích một phần ảnh hưởng của haematocrit đối với sai số hệ thống. Trong thẩm định lâm sàng, nồng độ thuốc trong mẫu DBS có tương quan tuyến tính khá chặt với nồng độ thuốc trong huyết tương (r^2=0.966). Hệ số góc của đường hồi quy là 1.49 (khoảng tin cậy 95% 1.32-1.77) cho thấy rằng moxifloxacin phân bố nhiều hơn trong tế bào máu so với huyết tương. Moxifloxacin cũng rất bền trong mẫu DBS. Không có sự phân hủy đáng kể nào tại điều kiện nhiệt độ thường sau ít nhất một tháng bảo quản.

Trong **chương 6a** và **chương 6b**, một phương pháp hiệu năng cao được xây dựng nhằm định lượng rifampicin, clarithromyicin và các chất chuyển hóa của chúng gồm 25-desacetyl rifampicin và 14 hydroxy clarithromycin trong mẫu DBS. Rifampicin là thuốc chống lao chủ lực với hoạt tính diệt khuẩn cao trên in vivo. Sự dao động cá thể về dược động học cũng như các tranh cãi hiện nay về liều tối ưu của thuốc này gợi ý rằng việc áp dụng TDM có thể có ích. Trước tiên, chúng tôi mô tả cách tiếp cận từng bước một nhằm loại bỏ hiệu ứng nhiễm chéo trong phương pháp được xây dựng và công bố trước đó. Việc thay cột sắc kí pha đảo C18 có gắn các nhóm phân cực bằng cột sắc kí pha đảo C18 thông thường đã loại bỏ được hiệu ứng này (**chương 6a**). Tiếp theo, chúng tôi áp kết quả này vào việc xây dựng phương pháp phân tích đối với mẫu phẩm DBS. Bằng việc pha thêm acid ethylenediaminetetraacetic (EDTA) và deferoxamin vào dung dịch chiết, "hiệu ứng matrix" do tương tác giữa rifampicin và các tạp chất nội sinh trong DBS được loại bỏ và hiệu năng xử lý mẫu được cải thiện. Kết quả trong thẩm định lâm sàng cho thấy mối tương quan cao giữa nồng độ trong DBS và huyết tương đối với rifampicin, clarithromycin và 14 hydroxy clarithromycin và gợi ý rằng kết quả phân tích từ DBS có thể được dùng để xác định nồng độ thuốc trong huyết tương. Thêm vào đó, các mẫu có nồng độ dưới ngưỡng định lượng cho kết quả giống nhau cho dù sử dụng DBS hay huyết tương. Do đó, việc lấy mẫu phân tích DBS có thể giúp xác định được các bệnh nhân không tuân thủ điều trị. Để đảm bảo sự tin cậy của kết quả lấy mẫu DBS ở các khu vực thiếu nguồn lực, độ ổn định của mẫu cần được đánh giá một cách toàn diện. Không giống như trong các mẫu dịch sinh học với độ ổn định thấp, rifampicin rất ổn định trong mẫu DBS (2 tháng ở nhiệt độ thường, 10 ngày ở 37°C và 3 ngày ở 50°C). Với kết quả đó, mẫu có thể được bảo quản và vận chuyển không cần các thiết bị làm lạnh (**chương 6b**).

Trong **chương 7**, việc ứng dụng phương pháp lấy mẫu và phân tích DBS vào giám sát nồng độ thuốc trong điều trị được mô tả chi tiết với linezolid. Phương pháp DBS được xây dựng và thẩm định có tính lặp lại. Thẩm định trên lâm sàng được thực hiện bằng cách so sánh nồng độ DBS và huyết tương bằng hồi quy Passing-Bablok và phân tích Bland-Altman. Tỷ lệ nồng độ trong DBS so với huyết tương là 1.2 (khoảng tin cậy 95% 1.12 – 1.27). Nồng độ thuốc trong DBS và plasma có độ tương hợp cao. Tương quan giữa chỉ số AUC_{0-24}/MIC có mối tương quan cao giữa DBS và plasma (Spearman rho=0.976, n=8). Điều này cho thấy việc giám sát nồng độ thuốc dựa trên phương pháp DBS sẽ tương tự như dựa trên phân tích mẫu huyết tương. Hơn nữa, linezolid ổn định trong mẫu DBS sau 2 tháng bảo quản ở 37°C và thậm chí ổn định ở nhiệt độ 50°C trong một tuần. Kết quả này gợi ý rằng việc điều chỉnh liều dùng để hạn chế độc tính và đảm bảo nồng độ điều trị là khả thi ngay cả ở các khu vực thiếu nguồn lực bằng việc áp dụng phương pháp lấy mẫu DBS.

Trong **chương 8**, chúng tôi bàn luận về vai trò của các kết quả nói trên đối với việc kiểm soát bệnh lao tại các nước đang phát triển. Vai trò của nhà thuốc tư nhân trong chiến lược chống lao của chương trình chống lao quốc gia tại Việt Nam được xác định và tìm hiểu. Yêu cầu cá thể hóa trong điều trị lao tại các nước đang phát triển sử dụng phương pháp DBS được bàn luận. Chúng tôi đề xuất mô hình mang tính hệ thống trong việc thực hiện giám sát nồng độ thuốc nhằm hỗ trợ quyết định lâm sàng trong điều trị lao tại các khu vực thiếu nguồn lực dựa trên phương pháp lấy mẫu và phân tích DBS.

SAMENVATTTING NEDERLANDS (DUTCH SUMMARY)

Wereldwijd is het streven het aantal doden door tuberculose (TB) terug te dringen tot de helft van het aantal in de periode 1990-2015. Het uiteindelijke doel is in 2050 de ziekte mondiaal volledig uit te bannen. Dat is niet gemakkelijk, omdat door de HIV epidemie en de toenemende resistentie van TB verwekkers voor de beschikbare antimicrobiële middelen, behandeling van TB steeds lastiger wordt.

In ***Deel 1*** van dit proefschrift wordt een veldstudie beschreven. Met behulp van pseudopatiënten werd aan een groot aantal medewerkers van openbare apotheken in Hanoi (Vietnam) vragen gesteld. Deze waren bedoeld om de kennis in algemene zin te toetsen en ook om de adviezen te vergelijken, die waren gegeven aan onze pseudopatiënten met symptomen die op TB leken. In **hoofdstuk 2** worden de details van dit onderzoek alsmede resultaten beschreven. Bijna de helft van de apotheek-medewerkers herkende de TB patiënt niet voldoende, althans zag geen reden hem door te sturen voor verdere diagnose en behandeling in een TB centrum. Een ander deel herkende de TB patiënt wel en stelde voor om bepaalde door de apotheek (zonder recept) te leveren antibiotica al vast te gaan innemen. Slechts 11% van de pseudo-TB-patiënten werden daadwerkelijk direct doorverwezen naar een TB centrum. De nationale TB-centra zijn gratis. In Vietnam kunnen apotheken een certificaat verwerven waarmee men aantoont dat men aan kwaliteitsprogramma's meedoet. Opmerkelijk was dat niet gecertificeerde apotheken de patiënt eerder naar een TB-centrum verwezen dan de gecertificeerde apotheken. In **hoofdstuk 3** is gekeken naar de beschikbaarheid van antituberculose middelen in apotheken in Vietnam. Hierbij werden zowel geneesmiddelen uit de eerste lijn maar ook de reserve antibiotica beoordeeld. De meeste apotheken beschikken slechts over één of twee antiTB middelen. Meestal is dit isonizide en/of rifampicine. Daarentegen beschikten de openbare apotheken dicht bij de gespecialiseerde tuberculoseklinieken over een groter assortiment anti TB middelen op voorraad. Een probleem is dat antibiotica die ook voor andere dan TB-infecties gebruikt kunnen worden, zonder recept worden verstrekt. Met name het gebruik van rifampicine voor open wonden was opvallend: 31% van de apothekers adviseerde dit middel voor deze indicatie. Nieuwe fluorochinolonen zijn tweede

keus middelen tegen resistente vormen van TB. Toch werden de fluorochinolonen door 36% van de apothekers of –medewerkers voor 'andere' infecties aanbevolen.

In ***Deel* 2** van het proefschrift staat de toepassing van een nieuwe methode voor het in bloed bepalen van verschillende antiTB-middelen centraal. Deze zogenaamde droge bloedvlek methode (Dry Blood Spot- DBS) stelt ons in staat geneesmiddelen te bepalen in slechts één druppel bloed, verkregen na een vingerprikje op filtreerpapier. De toepassing van deze DBS methode leent zich voor het bepalen van bloedspiegels van antiTB-middelen. Hiermee kan de behandelend arts of ziekenhuisapotheker nagaan of therapeutisch spiegels worden bereikt, de therapietrouw voldoende is en/of farmacogenetische variatie bij de bereikte bloedspiegel een rol speelt. Het voordeel van de DBS is dat men in niet stedelijke gebieden gemakkelijker bloed kan afnemen voor controle van de patiënten die anti-TB middelen moeten gebruiken. In **hoofdstuk 4** wordt een overzicht gegeven van de belangrijkste voor- en nadelen van de DBS methode. Bij de start van dit onderzoek werd de methode voor anti-TB-middelen nog niet, of op heel kleine schaal toegepast. Dit proefschrift geeft de aanzet tot uitgebreide toepassing van DBS bij patiënten met TB. In **hoofdstuk 5** wordt de DBS methode voor moxifloxacine beschreven, een middel dat inmiddels bij multiresistente TB in fase 3 trials wordt getest. De vereiste analytische DBS-methode ontwikkeling nam veel tijd in beslag, omdat er verschillende parameters die van invloed zijn op de reproduceerbaarheid van de methode, - onderzocht moesten worden. Zo is gekeken naar de invloed van de hoeveelheid rode bloedcellen (hematocriet) , het volume van de druppel bloed en de stabiliteit van de moxifloxacine in een DBS bij verschillende temperaturen en vochtigheidcondities. Het bleek dat moxifloxacine zich in de rode bloedcel ophoopt. De droge druppel bloed met moxifloxacine bleek tenminste 1 maand na afname nog bruikbaar voor analyse. In **hoofdstuk 6 en 7** is gekeken naar het ontwikkelen van een methode voor de bepaling van meerdere anti-TB-middelen in één DBS. Rifampicine is een middel dat vrijwel door alle TB-patiënten wordt gebruikt. In de eerste opzet werd gekeken naar een combinatie rifampicine met clarithromycine en de respectievelijke metabolieten 25-desacetylrifampicin en 14-hydroxychlarithromycine in DBS. Grote intra- en interindividuele variatie in farmacokinetiek van beide middelen rechtvaardigt het ontwikkelen van een DBS methodiek. Eerst in vitro en later ook voor in vivo monsters. Door toevoegen van Na-edetaat als metaalcomplexvormer, lukte het om het matrix effect tussen rifampicine en endogene componenten in de DBS te voorkomen. Hiermee kon een reproduceerbare methode voor rifampicine en clarithormcyne en metabolieten worden ontwikkeld. Rifampicine is in vloeibare media (plasma/serum/bloed) niet stabiel, maar in de 'droge' DBS vorm wel. In **hoofdstuk 8** is een DBS methode ontwikkeld voor linezolid, een nieuw veelbelovend antibioticum dat bij TB inmiddels is toegepast . De analysemethode is goed reproduceerbaar en de stabiliteit van de DBS is tenminste 2 maanden bij 37 °C. In **hoofdstuk 9** worden de resultaten van het onderzoek op de toekomstige positie van openbare apotheken in het nationale TB programma in Vietnam besproken. Voorts wordt nader ingegaan op de mogelijke impact van onze resultaten op de verdere ontwikkelingen binnen de behandeling van TB. Inmiddels zijn er voldoende klinische monsters bestudeerd en vergeleken met een klassieke bepalingsmethode, om de toepassing van DBS als methode voor Therapeutic Drug Monitoring te rechtvaardigen.

Toepassing van de DBS methode zal therapie op maat bij de tuberculosepatiënt mogelijk maken en daarmee een bijdrage leveren aan de slogan uit het WHO-Millenium Dossier 2050: "Help TB de wereld uit".

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Publication list

Vu DH, Koster RA, Wessels AMA, Greijdanus B, Alffenaar JWC, Uges DRA. Troubleshooting carry-over of LC-MS/MS method for rifampicin, clarithromycin and metabolites in human plasma. Journal of Chromatography B, 2013 Feb 15;917-918:1-4.

Vu DH, Bolhuis MS, Koster RA, Greijdanus B, de Lange WC, V Altena R, Brouwers JR, Uges DR, Alffenaar JW. Dried blood spot analysis for therapeutic drug monitoring of linezolid in MDR-TB patients. Antimicrob Agents Chemother. 2012 Nov; 56(11):5758-5763.

Vu DH, Rein N, Cobelens FG, Nguyen TTH, Le VH, Brouwers JRBJ. Suspected tuberculosis case detection and referral in private pharmacies in Vietnam. Int J Tuberc Lung Dis. 2012 Dec; 16(12):1625-1629.

Vu DH, Koster RA, Alffenaar JWC, Brouwers JRBJ, Uges DRA. Determination of moxifloxacin in blood using dried blood spots analysis. Br J Clin Pharmacol. 2011 Nov; 72(5): 861-861.

Vu DH, Alffenaar JW, Edelbroek PM, Brouwers JR, Uges DR. Dried blood spots: a new tool for tuberculosis treatment optimization. Curr Pharm Des 2011; 17(27):2931-2939.

Vu DH, Koster RA, Alffenaar JW, Brouwers JR, Uges DR. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. J Chromatogr B Analyt Technol Biomed Life Sci 2011 May 1; 879(15-16):1063-1070.

Nguyen TVA, Vu DH, Nguyen THM, Lachaux A, Boulieu R. Relationship between azathioprine dosage and thiopurine metabolites in pediatric IBD patients: Identification of covariables using multilevel analysis. Ther Drug Monit. 2013 Apr;35(2):251-7.

Tu HA, Vu HD, Rozenbaum MH, Woerdenbag HJ, Postma MJ. A review of the literature on the economics of vaccination against TB. 2012 Mar; 11(3):303-317.

Vu DH, Hoang KH, Ta MC (2007). A study on the antihypertensive effect and the safety of carvedilol in Bach Mai hospital. Vietnamese Pharmaceutical Journal 2007; 47(11): 3 - 8 (in Vietnamese).

Hoang KH, Ngo CD, Vu DH. Comparative examination of some online software for checking drug interactions. Vietnamese Pharmaceutical Journal 2007; 47(10): 3 - 8 (in Vietnamese).

Vu TT, Vu DH. Reality of the use of glucocorticoids before hospitalization and resulting ADR in patients in the Rheumatology Department of Bach Mai hospital. Vietnamese Pharmaceutical Journal 2007; 47 (8): 5 - 9. (in Vietnamese)

About the Author

Vu Dinh Hoa was born in a suburban village of Hanoi, Vietnam. He spent his childhood in the countryside. At the age of eight years old he moved with his family moved to Hanoi City. He grew up in the developing Hanoi City since then. In 2003, he gained his Bachelor degree at Ha Noi University of Pharmacy after finishing the thesis at the Department of Pharmaceutics. He continued his research and teaching within the Department of Clinical Pharmacy. He participated into several projects of the Department, which mainly focused on Bioequivalence and Bioanalysis. During this time, he also followed a Master program in Clinical Pharmacy at Hanoi University of Pharmacy. Since 2008, he has pursued his PhD study at the University of Groningen granted by Nuffic project in "Strengthening the Clinical Pharmacy Education at University Level in Viet Nam" (NPT VNM 240). His research topic emphasizes on "Clinical Pharmacology Aspects of Tuberculosis". With the supervision of Prof. J.R.B.J. Brouwers and Prof. VH Le, he conducted a field research on the private pharmacies in Vietnam in terms of tuberculosis control. He joined the research group of the Department of Hospital and Clinical Pharmacy, University Medical Center Groningen with the supervision of Prof. J.R.B.J. Brouwers, Prof. D.R.A. Uges and Dr. J.W.C. Alffenaar. In collaboration with many researchers in this group, he set up first studies on Dried Blood Spot analysis of antituberculosis drugs using LC-MS/MS. His research of interest is clinical pharmacokinetics and pharmacoepidemiology. He gained "PhD Top Publication" awards from Graduate school for Health research SHARE for two of his research articles. He is now working as a faculty member and a researcher in Hanoi University of Pharmacy (Viet Nam).

Vu Dinh Hoa is married with Nguyen Minh Hai and they have a son, Vu Dinh Minh Triet (Zin). They are now living in Hanoi.

Presentations

Vu DH, Rein N, Cobelens FG, Nguyen TTH, Le VH, Brouwers JRBJ. Suspected tuberculosis case detection and referral in private pharmacy in Vietnam Presented in FIP 2012, 5st – 8rd October, Amsterdam, The Netherland.

Vu DH, Bolhuis MS, Koster RA, Greijdanus B, de Lange WCM, V Altena R, Brouwers JRBJ, Uges DRA, Alffenaar JWC. Simultaneously determination of rifampicin, clarithromycin and metabolites in dried blood spots using LC-MS/MS presented at FIGON 2012, 1st-3rd October, Lunteren, The Netherlands.

Vu DH, Bolhuis MS, Koster RA, Greijdanus B, de Lange WCM, V Altena R, Brouwers JRBJ, Uges DRA, Alffenaar JWC. Linezolid in multidrug-resistant TB: individualized therapy feasible by dried blood spot analysis presented at FIGON 2012, 2nd October, Lunteren, The Netherlands.

Vu DH, Bolhuis MS, Koster RA, Greijdanus B, de Lange WCM, V Altena R, Brouwers JRBJ, Uges DRA, Alffenaar JWC. Dried blood spots for TB treatment optimization. Present at the 5th workshop on Clinical Pharmacology of Tuberculosis Drugs, 08th September 2012, San Francisco, US

Vu DH, Koster RA, Alffenaar JWC, Brouwers JRBJ, Uges DRA. Determination of moxifloxacin in blood using dried blood spots analysis. Presented at the FiGON day, 4th October 2010, Lunteren, The Netherlands. The abstract was published on Br J Clin Pharmacol November 2011

Vu DH, Rein N, Cobelens FG, Nguyen TTH, Le VH, Brouwers JRBJ. Clinical pharmaceutical aspects of tuberculosis in Vietnam. Invited lecture, 7th September 2010, KNCV, The Hague, The Netherlands.

Nam PTP, Vu DH, NTL Huong, Luyen L. Plasma concentrations of Rifampicin, Isoniazid and Pyrazinamide in patients with tuberculosis pleural effusion. Presented at conference Optimization of inhaled tuberculosis therapies and implications for Host-pathogens interaction, 3-5th November 2009, New Delhi.

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((co-) supervisors are between brackets)

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(prof K Taxis, prof D Wiersma)

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(prof FM Haaijer-Ruskamp, prof PA de Graeff, dr PGM Mol, SMJM Straus)

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(prof JGM Rosmalen, prof J Ormel, prof P de Jonge)

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Horst PGJ ter. *Clinical pharmacology of antidepressants during pregnancy*
(prof B Wilffert, prof LTW de Jong-van den Berg)

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